REMARKS

Upon entry of this amendment, Claims 8, 9, 11, 15-20, 23, 25, 29, 30, 32, 33, 35, and new Claim 36 constitute the pending claims. Claims 1-7, 10, 12-14, 21, 22, 24, 26-28, 31, and 34 are canceled without prejudice. Applicants reserve the right to prosecute claims of identical or similar scope to the canceled claims or claims prior to amendment in future continuation or divisional applications.

Applicants have amended the claims and added new Claim 36 to further clarify the subject matter claimed. Support can be found throughout the specification, including the original claims. *See*, for example, the first paragraph of page 1, the last paragraph of pages 2 and 3, *etc.*

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the Office Action.

Claim rejections under 35 U.S.C. § 112, first paragraph - enablement

Claims 8-11, 15-20, 23, 25, 29, 30, and 32-34 are rejected under 35 U.S.C. § 112, first paragraph, because the specification allegedly "(enables methods) for assessing feeding and/or weight gain pattern or diagnosing obesity in an individual in need thereof..., does not reasonably provide enablement for the claims as broadly recited."

Applicants respectfully disagree.

Evidently, the claimed invention is of commercial relevance to the medical/diagnostic industry, in that the measurement of MSH peptides is designed to be a simple quantitative measurement of the peptides in a biological fluid, from which the ratio of the specified MSH peptides can be calculated. As such, we direct the Examiner's attention to the specification at page 12, line 23 onwards for support, where Applicants have provided reference to methods known to those skilled in the art, of measuring/assaying for the relevant MSH peptides in a subject's plasma or other biological fluids. Using these techniques, the ratio of MSH peptides can be readily calculated according to the instant invention, for predicting/assessing risk of developing obesity.

Given that the claims have been amended to clarify that it is the ratio of desacetyl-α-MSH/α-MSH that is calculated, Applicants assert that there is no ambiguity in enablement of the current invention. The specification at page 51 and Example 2 at page 18 further provides support that it is the desacetyl-α-MSH/α-MSH ratio that is relevant in the claimed invention.

Applicants now address each of the enablement rejections in the sections below.

a. Scope of Enablement for "sample"

First of all, the Examiner appears to be concerned with the scope of the "sample" recited in the claims. On one hand, the Examiner does acknowledge Applicants' previous arguments that the specification must be taken as being in compliance with the enablement requirement unless there is reason to doubt the objective truth of the statements contained therein. On the other hand, the Examiner argues that it is unclear whether a measured ratio of desacetyl-α-MSH/α-MSH from an amniotic fluid sample or a cord blood sample is indicative of obesity or feeding problem in the mother of the fetus.

While this concern is not entirely without basis, Applicants submit that the concern at best relates to a clarification inquiry, but does not amount to the requisite reasonable doubt to the objective truth of the statement in the specification (*e.g.*, amniotic fluid or cord blood samples may be used to measure ratio of desacetyl-α-MSH/α-MSH). Applicants submit herewith published literature describing the measurement of desacetyl-α-MSH and/or α-MSH in cord blood (Facchinetti *et al.*, Am. J. Obstet. Gynecol. 161: 1267-1270, 1989, **Exhibit A**) and amniotic fluid (Mauri *et al.*, J. Endocrinol. Invest. 11: 345-349, 1988, **Exhibit B**), to provide evidence that such measurements can be done by methods well-known in the art.

Specifically, regarding cord blood, the literature analyzed α-MSH and desacetyl-α-MSH levels in maternal blood and cord blood, which are representative of the mother and fetus, respectively (*see* Figure 1 of **Exhibit A**). Thus the measurement of the ratio of the peptides is possible for both the mother and the fetus, and the ratio can be indicative of each, depending on whether the mother (maternal blood) or the fetus (cord blood) is being measured.

Regarding amniotic fluid, Mauri *et al.* suggest that desacetyl-α-MSH is the most abundant α-MSH-like peptide in the fetal pituitary gland, and therefore suggest that the fetal

pituitary is the main source of desacetyl-α-MSH in amniotic fluid. Thus the measurement of the ratio of desacetyl-α-MSH and α-MSH in amniotic fluid is also possible, and the ratio would be indicative of the status of the fetus.

The Examiner also argues that Applicants have not provided evidence concerning other biological samples, and it is allegedly "not plausible that any body tissue or fluid could be used to carry out the claimed methods."

Applicants hereby provide additional evidence to support the broad spectrum of tissues/samples from which desacetyl-α-MSH and/or α-MSH levels can be measured. Based on such measurements and the teaching of the instant application, one of skill in the art can easily calculate the ratio of desacetyl-α-MSH / α-MSH.

For example, α-MSH peptide measurement has been observed in both the epidermis and dermis tissues (Wakamatsu *et al.*, Pigment Cell Research 10: 288-297, 1997, **Exhibit C**). In another study, Kim *et al.* (Experimental Dermatology 16: 104-109, 2006, **Exhibit D**) reported immunohistochemical measurement of α-MSH and other peptides in various types of psoriatic lesions and control skin. Millington *et al.* (Peptides 5: 841-843, 1984, **Exhibit E**) reported measurement of immunoreactive α-MSH and ß-endorphin in microdissected regions of rat brain. In another study (Wilson *et al.*, Peptides 5: 681-685, 1984, **Exhibit F**), quantification of the permeability of the blood-cerebrospinal fluid barrier to α-MSH was also examined; both peptides measurable in the blood and cerebrospinal fluid.

Therefore, Applicants have presented overwhelming evident that the level of desacetyl-α-MSH and α-MSH can be measured in many different tissue types and samples. In contrast, other than a rather speculative conclusive statement ("is not biologically plausible that any body tissue or fluid could be used") not supported by any scientific literature, the Examiner has not established a reasonable doubt regarding the objective truth of the statements contained in the instant specification, and thus the specification must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); MPEP2164.04.

If the Examiner wish to maintain this rejection, Applicants respectfully invite the

Examiner to either cite scientific literature or provide a Declaration based on personal knowledge to support the rejection.

Applicants wish to reiterate that the initial burden is not on the Applicants to prove that each and every tissue sample can be used for the claimed invention. Rather, "...the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) ... 'it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure.' 439 F.2d at 224, 169 USPQ at 370" (emphasis added). MPEP2164.04.

The Examiner also argues that claim 25 makes reference to an *in vitro* cell, organ or tissue sample. Applicants wish to clarify that these tissues are not recited in claim 25. Although claim 29 does make reference to these tissues, it does so in the context of a "biological response system" as described on page 7 of the specification, wherein such a system is capable of responding to melanocortin peptides.

b. "Predicting" or "Assessing" Risks in Non-obese Subjects

The Examiner then raises some new concerns not previously addressed. Specifically, the Examiner argues that claims 15 and 16 read on "predicting" or "assessing" risk in apparently non-obese subjects, and that Applicants have not provided data to support this embodiment in the specification or in the literature. The Examiner argues that there is no evidence that the methods can be used to "predict risk" in non-obese subjects.

The Examiner appears to have based her enablement argument solely on the basis of the presence or absence of working examples. Essentially, the Examiner argues that only claims supported by actual data are enabled, and those without data support are not. This position clearly contradicts the enablement case law.

MPEP 2164.02 states that "[c]ompliance with the enablement requirement of 35 U.S.C.

112, first paragraph, does not turn on whether an example is disclosed. ... An applicant need not have actually reduced the invention to practice prior to filing. In *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ 2d 1302, 1304 (Fed. Cir. 1987), ... the Court held that 'The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it.' 822 F.2d at 1078, 3 USPQ2d at 1304 (quotation omitted)." (emphasis added)

Applicants again wish to remind the Examiner that the enablement requirement is assessed based on the totality of all evidence. The presence or absence of working example is merely one of the eight *Wands* factors that may be considered in assessing enablement. It should not *in itself* be the *sole* determinative factor for claim enablement or lack thereof.

Furthermore, in making the enablement rejection, the initial burden is on the Examiner to raise a reasonable doubt as to the objective truth of the statement in the specification. That burden is not satisfied simply by pointing out that Applicants have not provided working example to support enablement. Therefore, this rejection appears to fall short of the requisite legal standard, and Applicants respectfully request the Examiner to reconsider and withdraw the rejection.

Lastly, Applicants submit that the measurement of the ratio of desacetyl-α-MSH and α-MSH peptides to predict predisposition to developing obesity is a novel and non-obvious invention by Applicants. The absence of relevant data in the literature attest to the novelty and non-obviousness of the claimed invention.

c. *In vitro / In vivo* Correlation

Although Applicants have not provided clinical data in the instant specification with respect to human subjects, Applicants submit that such clinical data is not required to support the enablement of the claims, or even the enablement of embodiments concerning human subjects. As one of skill in the art would appreciate, human clinical trials often span years if not a decade or more, and it would be unreasonable to require or expect Applicant to have such data to support enablement. Partly for that reason, relevant case law merely requires a "reasonable correlation" between *in vitro* experiments (or animal model) and *in vivo* disease condition for the

purpose of satisfying the enablement requirement. *See* MPEP 2164.02:

> "Correlation" as used herein refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. ... if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications).
>
> ...
>
> A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):
>
> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (citations omitted)

Therefore, the correct analysis, based on this standard, should be whether the art recognize a particular *in vitro* / animal model as correlating to a specific disease condition. If so, then the Examiner should accept the model as correlating, unless the Examiner can come up with evidence that the model does not correlate. In addition, a rigorous or an invariable exact correlation is not required under *Cross v. Iizuka*. All that is required is a "reasonable correlation."

Applicants note that the Examiner has not provided any evidence to show that the animal model described in the specification does not reasonably correlate with obesity or imbalance in energy homeostasis, *etc.*, thus the Examiner has not carried the burden to support the non-enablement rejection.

d. "Increase" in the Ratio

The Examiner also had concerns regarding the terms "imbalance" and "disturbance" in claims 8 and 16 (also claim 15).

Applicants submit that the measurement of the ratio of the two peptides could indeed encompass underfeeding and weight loss. However, Applicants' present interest lies in predicting obesity. Therefore, solely in the interest of advancing prosecution, Applicants have amended the claims to recite that an increase in the ratio is "indicative" of metabolic disorders leading to obesity.

e. Applicability for Prediction

Furthermore, Applicants respectfully disagree with the Examiner that the claims only enable assessing an increase in feeding and/or weight gain pattern in an individual who is already obese. Applicants assert that the claimed methods are enabled for assessing an imbalance or disturbance in a non-obese individual, for essentially the same reasons as set out above, whereby the ratio of two factors can be examined in normal individuals and be indicative or predictive of potential disease development.

The Examiner boldly states that biochemical markers become evident "only as the condition manifest itself in the subject, not before," and cites Nakahara *et al.* (Biol. Psychiatry 2007) and Yarnell *et al.* (J. Epidimiol. Community Health 54: 344-348, 2000) to support this statement. Applicants respectfully disagree.

First of all, the Examiner cites Nakahara to support the conclusion that "biochemical markers of feeding behavior is evident once the condition is manifested." However, it is unclear how one can reach such a conclusion based on the data in Nakahara. It appears that Nakahara measured obestatin in a group of obese patients and a group of anorexic (AN) patients, and compared the levels to control patients. Contrary to the Examiner's assertion, obese patients have significantly lower levels of obestatin compared to control patients (page 2, left column, last paragraph, the 10th and 11th lines), while AN patients have significantly higher obestatin compared to control patients (not obese patients) (see same paragraph, the 4th to the 7th line). But these data says nothing about whether the level of obestatin can be *predictive* of future obesity, since the study is not designed to address this question.

Applicants hereby provide evidence to rebut the Examiner's conclusion that biochemical markers become evident "only as the condition manifest itself in the subject, not before."

For example, the ratio of LDL/HDL has been used in the art as a predictor of future cardiovascular disease risk in a normal subject (*see* Jukema *et al.*, Curr Med Res Opin. 21(11): 1865-1874, 2005, **Exhibit G**). Another study suggests that the ratio of apolipoprotein (apo) B/apoAI is a better index for risk assessment of coronary artery disease (Rasouli *et al.*, Clin Chem Lab Med. 44(8): 1015, 2006, **Exhibit H**). In a study of neuroblastoma (Sandler *et al.*, J Pediatr Surg. 37(3): 507-11, 2002, **Exhibit I**), it was found that the survivin/Fas ratio in primary tumors may be used to predict the risk for recurrent disease in patients with neuroblastoma. The S:F ratio appears to be a more sensitive predictor of recurrent disease than survivin expression alone. In another study (Onat *et al.*, Metabolism Clinical and Experimental 57: 207-214, 2008, **Exhibit J**), the role of serum C-reactive protein level was investigated as a risk factor in predicting metabolic syndrome (MS), hypertension, atherogenic dyslipidemia, type 2 diabetes mellitus and coronary heart disease. The results of the study showed that elevated levels of C-reactive protein is both an independent significant predictor and a risk factor of cardiometabolic risk pertaining to MS, hypertension, atherogenic dyslipidemia, diabetes and coronary heart disease.

These scientific publications are merely a small sample of publications that tend to refute the Examiner's assertion that biomakers can only be used as diagnostic markers in patients already having the disease, but cannot be used to predict future disease conditions in otherwise normal individuals. In fact, Yarnell (cited by the Examiner) also contradicts the Examiner's position by showing that BMI index at age 18 is predictive of future obesity in middle age.

Applicants also disagree with the Examiner's argument based on Yarnell. For one thing, Yarnell have not assessed and compared the instant claimed invention to the other obesity prognostic factors, and thus cannot be logically relied upon to show that the BMI index method is superior than the claimed invention. Further more, even assuming for the sake of argument that the BMI method is the most accurate predictor, it does not necessarily follow that no other methods can be accurate. The Examiner appears to argue for the untenable position that only the best method works, while all others cannot.

The Examiner also rejects claim 9 for lacking enablement on the same grounds argued above. Applicants have clarify the subject matter being claimed by amending claim 9 to recite

"... indicative of a predisposition to developing obesity." Applicants submit that all previous arguments related to predicting risk also applies to the rejection of claim 9.

The Examiner maintains the rejection to claim 31 on the basis of lacking enablement. While not acquiescing in the reasoning of the Office Action, Applicants have canceled claim 31 without prejudice to advance prosecution. Applicants reserve the right to prosecute claims of identical or similar scope in future applications.

Applicants respectfully request the Examiner to reconsider with withdraw all enablement rejections.

CONCLUSION

For the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of the pending rejections. Applicants believe that the claims are now in condition for allowance and early notification to this effect is earnestly solicited. Any questions arising from this submission may be directed to the undersigned at (617) 951-7000.

Applicants believe no fee other than those authorized in the accompanying Amendment Transmittal is due in connection with the filing of this response. If, however, any fee is due, please charge the fees to our Deposit Account No. **18-1945**, from which the undersigned is authorized to draw under order number **BSWV-P01-007**.

Dated: February 19, 2008

Respectfully submitted,

By_____

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Fetal intermediate lobe is stimulated by parturition

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The fetal pituitary gland secretes β-endorphin in blood in response to delivery. However, other forms of endorphin have recently been observed in the fetal pituitary, such as *N*-acetyl-β-endorphin, which is devoid of opiate activity, and a desacetylated form of α-melanocyte–stimulating hormone. Both endorphins originate in the pituitary intermediate lobe. The sensitivity of this lobe to labor stress was assessed by the evaluation of β-endorphin, *N*-acetyl-β-endorphin, melanocyte–stimulating hormone, and desacetylated α-melanocyte–stimulating hormone in maternal plasma and cord blood in 11 cases of vaginal delivery and 10 cases of elective cesarean section without labor. Plasma peptide levels were determined by specific radioimmunoassays after extraction on Sep-Pak C-18 cartridges and high-performance liquid chromatography fractionation. Cord blood samples of infants delivered vaginally showed higher β-endorphin (8.5 ± 1.6 pmol/L, mean ± SE) and desacetylated α-melanocyte–stimulating hormone (13.6 ± 3.2 pmol/L) levels than those delivered by elective cesarean section (3.7 ± 0.8 and 4.2 ± 1.1 pmol/L, for β-endorphin and desacetylated α-melanocyte–stimulating hormone, respectively). *N*-acetyl-β-endorphin and α-melanocyte–stimulating hormone levels do not differ in relation to the mode of delivery. In maternal circulation β-endorphin levels were higher in those delivered vaginally (5.2 pm 1) than in women who had cesarean sections (2.5 ± 0.5 pmol/L), whereas no changes were found for the other peptides. In vaginal deliveries, the level of desacetylated α-melanocyte–stimulating hormone was higher in cord blood (13.6 ± 3.2 pmol/L) than in maternal plasma (6.5 ± 3 pmol/L); there were no significant differences with regard to the other peptides. Fetal and maternal levels of all the peptides were similar in cases of cesarean section. We conclude that parturition activates proopiomelanocortin peptide release from both the anterior and the intermediate pituitary lobe and that the fetus secretes the appropriate β-endorphin molecule, that is, the peptide able to bind opiate receptors. Concomitant secretion of desacetylated α-melanocyte–stimulating hormone may occur with adrenal androgen activation at birth. (Am J Obstet Gynecol 1989;161:1267-70.)

Key words: Cord blood, β-endorphin, α-melanocyte–stimulating hormone, stress, parturition

High levels of β-endorphin in cord blood at delivery have been determined by several authors.[1, 2] The fetal origin of this response to parturition was supported by our findings that increased β-endorphin levels are detectable in the newborn circulation until the twelfth hour of life, that is, for a period several times longer than the plasma half-life of this opioid.[2] The most important stimulants of fetal β-endorphin and β-lipotropin secretion have been shown to be hypoxia and acidosis accompanying labor stress.[3] Such a fetal response to stress agrees with the observation that β-endorphin and β-lipotropin were detected in the fetal pituitary from the early stages of development,[4] and corticotropin-releasing hormone stimulates peptide secretion from superfused fetal hemipituitaries from the time of midgestation.[5]

Recently we observed that different forms of β-endorphin and α-melanocyte–stimulating hormone (α-MSH) are present in the fetal pituitary: a desacetylated form of α-MSH[6, 7] (Des-Ac-α-MSH) and acetylated forms of β-endorphin, such as *N*-acetyl-β-endorphin (*N*-Ac-β-endorphin),[8] which is devoid of opiate activity.[9] *N*-Ac-β-endorphin represents 10% of the total β-endorphin immunoreactivity measured in the gland. This peptide is thought to originate, together with α-MSH, in the pituitary intermediate lobe from the cleavage of proopiomelanocortin (POMC). Indeed, α-*N*-acetyltransferase, the acetylating enzyme required for the posttranslational change, is absent in the anterior lobe.

In the rat, repeated acute and chronic foot shock stresses allow the secretion of POMC peptides from both the anterior and the intermediate lobes.[10] Because the opioid release is stimulated by labor stress and the fetal pituitary is characterized by the presence of a well-developed intermediate lobe, we investigated its possible activation at birth by measuring α-MSH and *N*-Ac-β-endorphin in cord blood.

Material and methods

A total of 15 ml of blood was collected from umbilical cord samples immediately after the extraction of the

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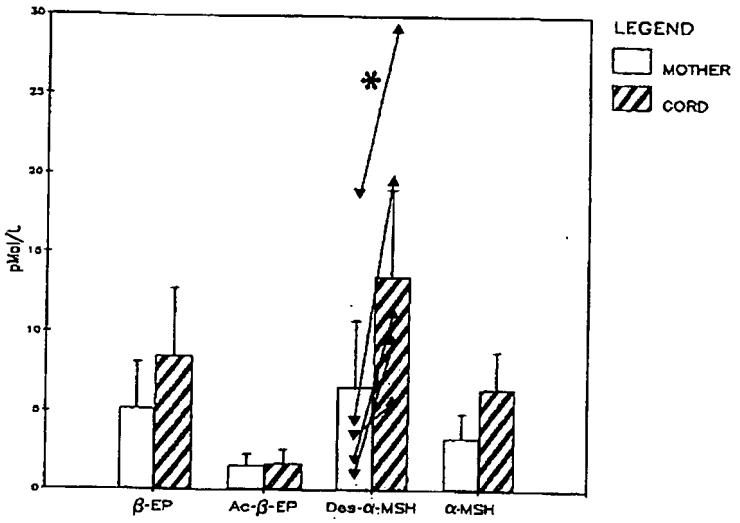

Fig. 1. Maternal and cord plasma peptide levels (mean ± SE) in spontaneous deliveries. *Solid lines* refer to paired maternal and cord samples. *Asterisk* indicates significant difference according to Mann-Whitney rank test.

baby in 10 cases of elective cesarean section in absence of labor and in 11 spontaneous vaginal deliveries. In five cases within each group paired maternal blood samples were collected. Further maternal samples were collected at delivery (six cases) or at the moment of fetal extraction (five cases). Parturition occurred at term in healthy pregnant women, and Apgar scores were >8. No obstetric complications were noted in spontaneous deliveries and no drugs were administered to the mothers. Elective cesarean sections were performed between 38 and 40 weeks of pregnancy because of previous cesarean section, and in the absence of uterine contractions.

Heparinized blood samples (with addition of 500 KU/ml of Trasylol) were centrifuged for 10 minutes at 1500 *g* and plasma was stored at −20° C until assays, which were done within 2 months. Each plasma sample (4 to 6 ml) was loaded onto activated Sep-Pak C-18 cartridges (Waters Assoc., Milford, Mass.); the column was then washed with 5 ml of 20% methanol in 0.5 mol/L acetic acid solution and the peptides were recovered from the column by washing with 4 ml of 90% methanol–acetic acid solution. The eluates were dried, redissolved in 300 μl of acetonitrile–0.01N hydrochloric acid (18:82) solution, and submitted to high-performance liquid chromatography (HPLC) fractionation as previously described.[7, 8]

β-Endorphin and α-MSH tagged with iodine were added to plasma samples and were extracted by the above conditions; recoveries were 91.9% ± 10.8% and 78.4% ± 7.1%, respectively.

The HPLC apparatus (Waters) was equipped with a reverse-phase C-18 μBondapack column, 3.9 × 300 mm, 10 μm particle size. The elution was carried out in a convex gradient, starting from 18% to 33% acetonitrile in 0.01N hydrochloric acid for 15 minutes, followed by a further increase of acetonitrile, which reached 36% in 10 minutes. Flow rate was 1.5 ml/min. Starting from the fourth minute of elution, 40 fractions were collected (every 30 seconds), dried, and dissolved in 500 μl of 0.12 mol/L phosphate buffer, pH 7.4, and 0.1% bovine serum albumin; immunoreactivity was tested through different radioimmunoassays (RIAs).

Anti-β-endorphin serum (obtained by Dr. A. E. Panerai, Milan) fully recognizes β-endorphin and β-lipotropin but does not react with 1-16 β-endorphin and 1-17 β-endorphin. Details of the RIA were previously reported.[11]

Antiserum α-MSH was supplied by Dr. V. Wiegant (Utrecht). It reacts 25% with Des-Ac-α-MSH and 100% with diacetyl-α-MSH. The RIA procedure was previously reported.[12] Antiserum against the C-terminal of *N*-Ac-β-endorphin was provided by Dr. J. Funder (Melbourne), and the RIA was previously described elsewhere.[13] This antiserum does not recognize β-endorphin, although it fully reacts with *N*-Ac-β-endorphin, 1-27 *N*-Ac-β-endorphin, 1-16 *N*-Ac-β-endorphin, and 1-17 *N*-Ac-β-endorphin.

Five plasma samples were added (1 ng each of *N*-Ac-β-endorphin, β-endorphin, and α-MSH); they were then submitted to the above-reported purification procedure and RIAs. Recoveries were 84.1% ± 10.4% for *N*-Ac-β-endorphin, 81.9% ± 11.6% for β-endorphin, and 65.6% ± 8.9% for α-MSH. Data were not corrected for those recovery losses.

Statistical analysis was performed with analysis of var-

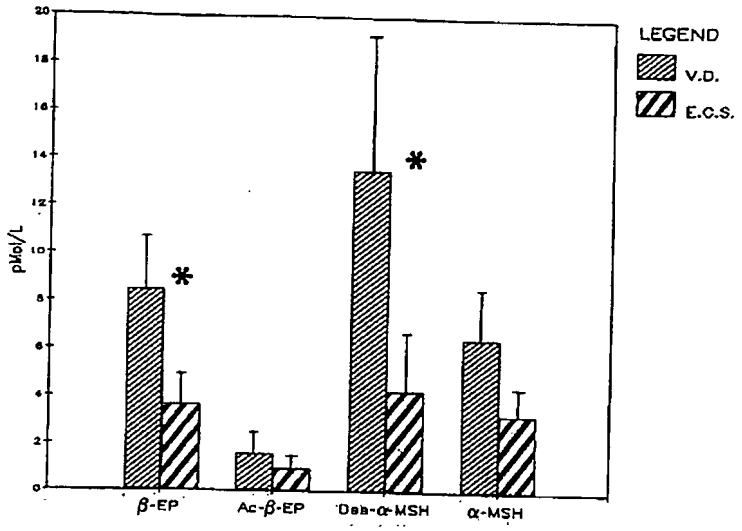

Fig. 2. Plasma peptide levels (mean ± SE) in umbilical cords of vaginally delivered infants *(VD)* and in newborns delivered by elective cesarean section *(ECS)* in absence of labor. *Asterisks* indicate significant differences by means of ANOVA.

iance and both Mann-Whitney and Wilcoxon rank sum tests, when appropriate. Correlation coefficients were calculated by the least-squares method.

Results

HPLC in combination with RIA was used to ascertain that β-endorphin, α-MSH, *N*-Ac-β-endorphin, and des-Ac-α-MSH circulate in both maternal and umbilical cord blood. When all data of spontaneous delivery are considered, des-Ac-α-MSH levels measured in the cord blood (13.6 ± 3.2 pmol/L, mean ± SE) were higher than those found in the maternal circulation (6.4 ± 3 pmol/L; $p = 0.019$, Mann-Whitney) (Fig. 1). Analysis of the data, taking into account only paired samples, confirms this finding (cord, 16.8 ± 5.8 pmol/L; mother, 7.2 ± 4.2 pmol/L; $p = 0.061$, Wilcoxon rank sum test.) The concentrations of the remnant peptides were similar between mothers and fetuses. In cases of elective cesarean section, maternal and fetal concentrations of all the peptides were similar.

In a comparison with cord samples, it appears that infants delivered vaginally have significantly higher plasma β-endorphin levels (8.5 ± 1.6 pmol/L) than those delivered by cesarean section (3.7 ± 0.8 pmol/L; $p < 0.01$) (Fig. 2). The same is true for Des-Ac-α-MSH; concentrations in infants delivered vaginally (13.6 ± 3.2 pmol/L) were three times higher than in infants in the cesarean section group (4.2 ± 1.1 pmol/L; $p < 0.05$). No differences between the two groups were observed for levels of α-MSH and *N*-Ac-β-endorphin.

With regard to maternal circulation, only β-endorphin showed a significant difference in relation to the mode of delivery. The values of the vaginal delivery group (5.2 ± 1.1) were higher than those in the cesarean group (2.5 ± 0.5 pmol/L; $p < 0.01$).

Independently of the mode of parturition, a significant correlation between β-endorphin and Des-Ac-α-MSH levels was found in cord blood ($r = 0.72$, $p < 0.01$).

Comment

These data add to the knowledge that several POMC-derived peptides circulate in maternal and cord plasma at parturition.[1-3] In addition to β-endorphin and α-MSH, *N*-Ac-β-endorphin and Des-Ac-α-MSH have been measured, in agreement with the observation that the fetal pituitary contains all these hormones from early gestation.[4-6]

In cases of vaginal delivery, our data confirm that β-endorphin rises at birth in both the maternal and the fetal circulation, thus reinforcing the concept that parturition is a stressful event for both mother and fetus.[1-3] This is further confirmed by the finding that parturition without labor, as in the case of elective cesarean section, is characterized by lower β-endorphin levels than are found in vaginal delivery.

In the fetal circulation, one tenth of β-endorphin is present in the form of acetylated β-endorphin. *N*-Ac-β-endorphin levels were not affected by parturition, and their values remain low both in the mother and in the fetus. It is well known that *N*-terminal acetylation impairs the ability of endorphins to bind opiate receptors.[9] These data therefore indicate that parturition activates only the secretion of opiate-active peptides. Interestingly, similar data have been reported in fetal lambs. Although *N*-Ac-β-endorphin in this species rep-

resents most of the total immunoreactivity of β-endorphin in the plasma, during experimental hypoxia only opiate-active β-endorphin was released.[14]

Concomitant with release of β-endorphin, parturition stimulates an important release of *N*-Ac-β-endorphin in cord blood but not in maternal blood. This desacetylated form of α-MSH (adrenocorticotropic hormone 1-13 NH_2) is thought to originate from the cleavage of adrenocorticotropic hormone in the intermediate lobes. In lower mammals this peptide is immediately acetylated and transformed into α-MSH. Thus, in view of the positive correlation between β-endorphin and *N*-Ac-β-endorphin levels in cord blood, the previous data led to the conclusion that a stimulation of the fetal intermediate lobes occurs at delivery. A similar finding was reported by us at midgestation. Prostaglandin-induced labor stimulates a pituitary accumulation of both adrenocorticotropic hormone and *N*-Ac-β-endorphin, suggesting a response of both anterior and intermediate lobes.[6, 7] α-MSH cord levels remain unaffected by parturition, whereas the concentrations of its desacetylated form increased. Thus the lack of an increase in *N*-Ac-β-endorphin and α-MSH at parturition demonstrates that the posttranslational acetylation of POMC peptides is of little importance in humans and is not activated by physiologic stress in the fetus.

Acetylation interferes with the biologic activity of peptides. Indeed, α-MSH shows more potent behavioral and melanotropic activities than does *N*-Ac-β-endorphin. Because the melanotropic function lost its importance in humans, it is probable that pituitary enzymes changed accordingly, shifting to synthesis of the desacetylated compound.

However, little information exists with regard to the biologic effects of *N*-Ac-β-endorphin. Its fetal pituitary contents increase in the first half of the second trimester,[6] concomitant with the adrenal sprout.[15] Moreover, the pharmacologically induced increase of Des-Ac-α-MSH in the dog pituitary led to the histologic and functional maturation of the adrenal zona reticularis, without apparent changes in pituitary adrenocorticotropic hormone or β-endorphin contents but with a reduction of α-MSH.[16] This inner portion of the adrenal cortex secretes the same Δ^5 androgens that characterize the zona fetalis of the adrenal. We could speculate that Des-Ac-α-MSH, instead of the previously proposed α-MSH,[15] is the peptide that controls adrenal androgen secretion during fetal life, perhaps functioning as a competitive antagonist to α-MSH.

In conclusion, these data demonstrate: (1) that parturition activates POMC peptide release from both the anterior and the intermediate pituitary lobe, (2) that the fetus reacts to labor stress with a release of the appropriate β-endorphin molecule (i.e., a peptide able to bind opiate receptors and therefore enhance analgesia), and (3) that the concomitant secretion of Des-Ac-α-MSH may be related to adrenal androgen activation at birth.

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Des-acetyl-α-MSH and not α-MSH is the major form of α-MSH in amniotic fluid

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ABSTRACT. Immunoreactive α-melanocyte stimulating hormone (IR-α-MSH)-like activity was measured by radioimmunoassay (RIA) in at term pregnancy amniotic fluid prior and after adsorption on a Sep-pak C18 cartridge. α-MSH activity was 3-4 times lower after Sep-pak purification but, unlike the levels of IR-α-MSH in the fluid analyzed *in toto*, increased linearly with the volume of fluid analyzed. Furthermore, fractionation by high pressure liquid chromatography (HPLC) revealed that IR-α-MSH recovered from the Sep-pak was due to several peptides rather than to a single peptide. The most abundant of them (50% of total activity) behaved like authentic des-acetyl-α-MSH. Since des-acetyl-α-MSH is also the most abundant α-MSH-like peptide in the fetal pituitary gland, the present results suggest that the fetal pituitary is a main source of des-acetyl-α-MSH in the amniotic fluid.

INTRODUCTION

Adrenocorticotropin (ACTH) and α-melanocyte stimulating hormone (α-MSH) derive together with β-endorphin from the processing of a common 31,000 d precursor protein called proopiomelanocortin (POMC). This processing has been extensively characterized in the pituitary gland where POMC is cleaved to ACTH and β-lipotropin in the anterior lobe, while α-MSH and β-endorphin are mainly formed in the intermediate lobe (1, 2).

A similar processing is believed to occur also in the human fetal pituitary, that contains high amounts of α-MSH and endorphins (3, 4). However, the role of α-MSH in the fetus is unknown. It has been suggested that this hormone could play a role in the fetal growth (5), since α-MSH has been found to be capable of restoring fetal growth in encefalectomized rats (6). Moreover, a regulation of fetal adrenal cortex by α-MSH during early and late pregnancy in several animal species, including human, has been suggested, but at present conflicting reports exist on this purpose (7-9). Recently immunoreactive α-MSH material (IR-α-MSH) has been determined also in the human amniotic fluid (10) and it has been speculated that it could be considered as a marker of fetal distress (19). However very little is known about the nature of IR-α-MSH-like material found in the amniotic fluid. In order to better characterize human amniotic α-MSH-like activity, we have fractioned by high pressure liquid chromatography (HPLC) the peptide extract from at term pregnancy amniotic fluids, analyzed the fractions by means of a specific radioimmunoassay (RIA) for α-MSH, and then compared the results with those obtained by RIA in amniotic fluid *in toto*.

Key words: α-MSH, amniotic fluid, radioimmunoassay, HPLC.

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MATERIALS AND METHODS

Amniotic fluid

Samples of amniotic fluid (30 ml) were collected by transabdominal amniocentesis performed to evaluate fetal lung maturity prior to repeat cesarean section from at-term pregnant women (37th to 40th week). Five pregnancies were considered to be normal on the basis of the following criteria: absence of complication before or during pregnancy, normal antropometric fetal parameters as determined by ultrasound, normal levels of human placental lactogen (HPL) and estriol in maternal blood, and normal lung maturity tests. In addition, the absence of fetal distress was monitored by cardiotocography. Of the remainder 4 pregnancies, 2 were complicated by diabetes and fetal macrosomy and the other 2 by hypertension associated to fetal retarded growth.

Isolation of α-MSH activity from amniotic fluid

From each sample of amniotic fluid 2 aliquots of 10 ml were transferred immediately after collection in polypropylene 15 ml tubes containing 10 IU of trasylol and frozen at −20 C until assayed. After centrifugation at 5000 rpm for 5 min, the supernatant was transferred to another polypropylene tube containing 1 ml of a mixture containing concentrated HCl, trifluoroacetic acid

(TFA) and formic acid (V:V:V) and 90 mg of NaCl. After vortexing the samples were centrifuged 5 min at 5000 rpm. The clear supernatant was passed 5 times on a Sep-pak C18 cartridge (Waters Ass.) previously washed with methanol and 01% TFA. The Sep-pak was then washed with 5 ml of 0.1 TFA and the peptides eluted with 3 ml of 0.1% TFA in 80% acetonitrile. Under these conditions more than 80% of ^{125}I-α-MSH added to amniotic fluid was recovered.

A similar recovery of 83%, 81%, 83% and 81.4% was obtained also by RIA after the processing 10 ng of cold MSH, des-acetyl-α-MSH α-MSH-sulfoxide and des-acetyl-α-MSH-sulfoxide, respectively. The eluate was then concentrated with a Speed Vac (Savant) and dissolved in buffer for RIA or in 0.1% TFA for further fractionation by HPLC.

For the determination of IR-α-MSH in amniotic fluid *in toto*, the fluid was centrifuged at 5000 rpm 10 for min. Aliquots of the clear supernatant were concentrated with a Speed Vac and dissolved in buffer for RIA.

HPLC

A Varian 5000 high pressure liquid chromatograph equipped with a variable wavelength UV detector and a 0.39 × 30 cm μBondapak C18 column (Waters Assoc.) was used. The mobile phase was made with two solvents, A and B. Solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in 80% acetonitrile. The gradient was linear from 20% to 40% B in 40 min, at a flow rate of 1 ml/min and room temperature. Under these conditions synthetic des-acetyl-α-MSH-sulfoxide, α-MSH-sulfoxide, des-acetyl-α-MSH, α-MSH and di-acetyl-α-MSH were well separated and eluted in a volume of 1-2 ml (Fig. 2). When amniotic α-MSH activity recovered from the Sep-pak was fractionated, 45 fractions of 1 ml were collected, concentrated with a Speed Vac and analyzed for α-MSH immunoreactivity.

MSH radioimmunoassay

α-MSH radioimmunoassay was performed by using a specific antibody raised in rabbit against synthetic α-MSH conjugated with hemocyanin and previously characterized (11). Briefly, the antibody cross-reacted 100% with des-acetyl-α-MSH, des-acetyl-α-MSH-sulfoxide and α-MSH-sulfoxide, 94% with di-acetyl-α-MSH, 10% with ACTH 1-16, ACTH 1-19, ACTH 1-24, and did not cross-react with ACTH 4-10, ACTH 11-24, ACTH 11-39 and with other several unrelated peptides. The antibody (final dilution 1:12,000) was incubated with 0.05 M sodium phosphate buffer pH 7.5 containing 0.5% bovine serum albumin and 10,000 cpm ^{125}I-α-MSH.

Iodinated ^{125}-α-MSH was prepared by means of the chloramine T method (12) and purified by gel filtration on a Sephadex G25. α-MSH was used as a standard. Bound and free ^{125}α-MSH were separated by adsorption into charcoal at room temperature. The linear range of sensitivity was from 1 to 100 pg.

Peptides

Synthetic α-MSH and des-acetyl-α-MSH were purchased from Peninsula Laboratories (CA, USA), di acetyl-α-MSH from Sigma. Des-acetyl-α-MSH-sulfoxide and α-MSH-sulfoxide were obtained by incubating the respective unoxidized peptides with H_2O_2 at 0°C for 30 min. The oxidized peptides were then purified by HPLC.

RESULTS

As shown in Table 1, the concentration of IR-α-MSH in amniotic fluid of all 9 patients prior adsorption on the Sep-pak C18 is 3-4 times higher than that eluted from the Sep-pak C18 (19.6 ± 1.3 versus 5.9 ± 0.8 pmol/l).

Table 1 - *Concentration of IR-α-MSH in at term amniotic fluid prior and after adsorbtion on a Sep pak C18.*

Patient no.	IR-α-MSH Prior Sep pak		After Sep pak	
	pg/ml	pmol/l	pg/ml	pmol/l
1[1]	30	18	12	7.2
2	32	19.2	14	8.4
3[2]	44	26.4	13	7.8
4[1]	24	14.4	5	3.0
5	28	16.9	7	4.2
6[2]	26	15.6	5	3.0
7	36	21.6	6	3.6
8	38	22.8	13	7.8
9	36	21.6	14	8.4
Mean ± SE	32.6 ± 2.1	19.6 ± 1.2	9.9 ± 1.3	5.9 ± 0.76

IR-α-MSH values are calculated by processing 1 ml of amniotic fluid prior and after Sep pak purification. Pregnancy complicated by [1]hypertension or [2]diabetes.

However, IR-α-MSH increased linearly with the volume of analyzed amniotic fluid only after Sep-pak purification (Fig. 1).

When IR-α-MSH recovered from the Sep-pak C18 was fractionated on the μBondapak C18, 5 immunoreactive peaks eluting at 14, 17, 21, 28 and 32 min, respectively, were found. These peaks showed the same retention times of authentic des-acetyl-α-MSH-sulfoxide (14 min), α-MSH-sulfoxide (17 min), des-acetyl-α-MSH (22 min), α-MSH (28 min), and di-acetyl-α-MSH (32 min), respectively. Some IR-α-MSH eluted also at 24-25 min, although not always, giving an apparent larger elution volume of IR-α-MSH peak eluting at 22 min (Fig. 2).

The concentration of all 5 immunoreactive forms of

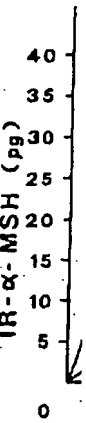

Fig. 1 - *IR-fluid prior Amniotic fl respective ml were co and analyz were adso TFA conta um, dissol the mean*

Fig. 2 - *HP tored by ad amniotic fl through a S 01% TFA a After conc was dissol Bapak C1 Solvent A = 80% aceto 0 min (flo collected, Of IR-α-M authentic des-acetyl rely. Cros line UV*

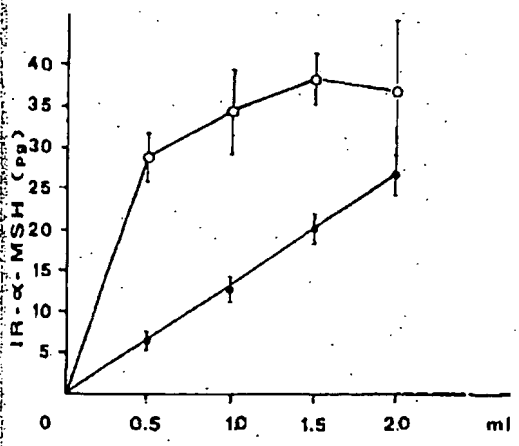

Fig. 1 - *IR-α-MSH-like activity in at term pregnancy amniotic fluid prior to (o) and after (●) recovery from Sep-pak C18. Amniotic fluid was pooled and aliquots of 0.5, 1, 1.5 and 2 ml, respectively, were prepared. Two aliquots of 0.5, 1, 1.5 and 2 ml were concentrated under vacuum, dissolved in RIA buffer and analyzed by RIA. Other 2 aliquots of 0.5, 1, 1.5 and 2 ml were adsorbed on a Sep-pak C18, eluted with 3 ml of 0.1% TFA containing 80% acetonitrile, concentrated under vacuum, dissolved in RIA buffer and analyzed by RIA. Each value is the mean ± SE of 3 experiments.*

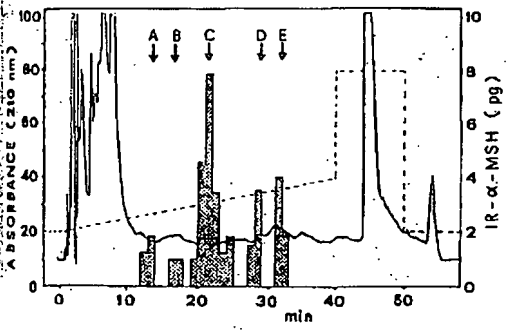

Fig. 2 - *HPLC fractionation of IR-α-MSH-like activity recovered by adsorption on Sep-pak C18 from at term pregnancy amniotic fluid. Ten ml of amniotic fluid were passed 5 times through a Sep-pak C18. The Sep-pak was washed with 5 ml of 0.1% TFA and eluted with 3 ml of 0.1% TFA in 80% acetonitrile. After concentration under vacuum (see Methods) the eluate was dissolved in 100 µl of 0.1% TFA and injected on a µBondapak C18 column monitored at 210 nm and eluted with Solvent A = 0.05% TFA in water and Solvent B = 0.05% TFA in 80% acetonitrile. The gradient was linear from 20% to 40% B in 40 min (flow rate 1 ml/min). Forty-five fractions of 1 ml were collected, concentrated, dissolved in RIA buffer and assayed for IR-α-MSH. A, B, C, D and E represent the retention time of authentic des-acetyl-α-MSH-sulfoxide, α-MSH-sulfoxide, des-acetyl-α-MSH, α-MSH and di-acetyl-α-MSH, respectively. Cross-hatched bars represent IR-α-MSH, the continuous line UV absorbance and the dotched line % of solvent B.*

α-MSH present in amniotic fluid, calculated assuming that they represent substances with an affinity for the antibody similar to that of α-MSH or des-acetyl-α-MSH, is reported in Table 2. The peaks corresponding to des-acetyl-α-MSH, α-MSH and di-acetyl-α-MSH represent 48%, 11% and 13%, respectively, of the total activity. The other 2 peaks account for the remainder 28% immunoreactivity. No difference in the amount of the various forms of IR-α-MSH among the 9 women was found, including the samples collected from pregnancies complicated by diabetes and by hypertension.

DISCUSSION

The present results confirm previous studies showing that IR-α-MSH is present in at term amniotic fluid (10). Indeed, when IR-α-MSH activity was measured in amniotic fluid *in toto*, the equivalent of 32 pg of α-MSH was found in 1 ml of amniotic fluid. This is in agreement with the above study (10) which reported 35-40 pg/ml of IR-α-MSH in at term amniotic fluid. However the concentration of IR-α-MSH material did not increase linearly with the volume of fluid analyzed. A possible explanation for these results is that unknown substances present in the amniotic fluid could interfere with our RIA. According to this hypothesis, the adsorption of α-MSH activity on Sep-pak C18, although decreasing IR-α-MSH levels to about 10 pg/ml (5.9 pmol/l), allowed the linear increase of IR-α-MSH concentration with the volume of analyzed fluid. This suggests that the concentration of α-MSH determined in amniotic fluid *in toto* is overestimated, particularly when small volumes of fluid are analyzed.

Moreover, our data show that IR-α-MSH-like activity recovered from the Sep-pak is due to an heterogeneous population of peptides rather than a single peptide. In particular, of the 5 immunoreactive peaks obtained by HPLC, the most abundant coelutes with authentic des-acetyl-α-MSH (48% of total immunoreactivity), while only 11% of the activity coelutes with authentic α-MSH. If one assume that the peaks eluting at 14 and 17 min represent the oxidized forms of the above peptides due to the extraction procedure, a very likely hypothesis, it would be concluded that about 63% of IR-α-MSH in amniotic fluid is des-acetylated α-MSH, 24% is authentic α-MSH and 13% is di-acetylated α-MSH.

These data indicate that des-acetyl-α-MSH and not α-MSH is the major form of α-MSH-like substance present in the amniotic fluid. Tilders et al. (13) and Brubaker et al. (14) have also found that des-acetyl-α-MSH is the major form of IR-α-MSH in the human fetal pituitary gland. The presence of des-acetyl-α-MSH in both fetal pituitary and amniotic fluid suggests that the pituitary gland might be a main source of amniotic des-acetyl-α-MSH. Thus α-MSH levels in the liquor might reflect the amount of α-MSH released by the

Table 2 - Concentration of the various forms of IR-α-MSH activity present in the amniotic fluid after adsorption on Sep pak C18 and fractionation by HPLC.

Patient no.	IR-α-MSH (pmol/l) eluting at min: 13-14	16-17	21-23	27-28	31-32	Total
1[1]	0.12	----	0.26	0.11	0.12	0.61
2	0.12	0.12	0.41	0.27	0.20	1.12
3[2]	0.06	0.06	0.48	0.23	0.13	0.96
4[1]	0.23	----	0.35	0.23	0.20	1.01
5	0.28	0.54	1.29	----	0.18	2.29
6[2]	0.36	0.27	0.55	----	0.11	1.29
7	0.37	0.18	0.66	----	0.07	1.28
8	0.38	0.50	1.42	0.43	0.51	3.24
9	0.18	0.12	1.21	0.30	0.36	2.17
Mean ± SE	0.23 ± 0.04	0.20 ± 0.06	0.74 ± 0.14	0.17 ± 0.05	0.21 ± 0.04	1.55 ± 0.26
% of total	15	13	48	11	13	100

Pregnancy complicated by [1]hypertension and [2]diabetes.

fetal pituitary itself. However, further experiments are necessary to clarify this point, since IR-α-MSH has been detected also in placental tissue (15) as well as in umbilical blood (15).

As to the possible significance of IR-α-MSH activity in amniotic fluid it is pertinent to recall that fetal α-MSH is considered to be involved in intrauterine growth and fetal brain development (16), as well as in the regulation of the pituitary-adrenal axis (7-9) and also in the stimulation of sebum secretion by fetal skin cells (18). Recently, increasing β-endorphin levels in amniotic fluid were found to be associated with complicated pregnancies (10). In contrast, the above study failed to show any correlation between α-MSH levels in amniotic fluid and pathological maternal and/or fetal abnormalities. However, the above results need to be confirmed by performing RIA after purification of amniotic IR-α-MSH, in view of the lack of linearity we have found by analyzing amniotic fluid *in toto*. With regard to a possible role of fetal α-MSH in fetal growth, a recent report (19) failed to show any correlation between α-MSH concentrations in umbilical blood and fetal growth. The above authors found a small but significant increase in α-MSH levels in the umbilical blood of fetuses from mother suffering from diabetes mellitus. However, it has still to be ascertained if in umbilical blood α-MSH derives from the fetus or also from the placenta.

Finally, evidence that α-MSH exerts behavioral effects via a direct action on the central nervous system, put foward an intriguing aspect of investigation concerning a possible correlation between fetal behavioral states and endocrine milieu (20).

In conclusion, the present results show that several forms of IR-α-MSH are present in the at term amniotic fluid and that the most abundant of them behaves like authentic des-acetyl-α-MSH.

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Exhibit C

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Original Research Article

Characterisation of ACTH Peptides in Human Skin and Their Activation of the Melanocortin-1 Receptor

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α-Melanocyte-stimulating hormone (α-MSH) is a proopiomelanocortin (POMC)-derived peptide, which is produced in the pituitary and at other sites including the skin. It has numerous effects and in the skin has a pigmentary action through the activation of the melanocortin-1 (MC-1) receptor, which is expressed by melanocytes. Recent evidence suggests that the related POMC peptides such as adrenocorticotrophin (ACTH), which is the precursor of α-MSH, is also an agonist at the MC-1 receptor. By using immunocytochemistry, we confirmed the presence of α-MSH in human skin where staining was evident in keratinocytes and especially strong in melanocytes and possibly Langerhans cells. ACTH was also present and tended to show the strongest reaction in differentiated keratinocytes. Immunostaining was also observed for the prohormone convertases, PC1 and PC2, which are involved in the formation of ACTH and its cleavage to α-MSH, respectively. The amounts of immunoreactive ACTH exceeded those of α-MSH. Using HPLC we identified for the first time the presence of ACTH1-39, ACTH1-17, ACTH1-10, acetylated ACTH1-10, α-MSH, and desacetyl α-MSH in epidermis and in cultured keratinocytes. The ability of these peptides to activate the human MC-1 receptor was examined in HEK 293 cells that had been transfected with the receptor. All peptides increased adenylate cyclase in these cells with the following order of potency: ACTH1-17 > α-MSH > ACTH1-39 > desacetyl α-MSH > acetylated ACTH1-10 > ACTH1-10. ACTH1-17 also increased the dendricity and melanin content of cultured human melanocytes indicating that the peptide was able to activate MC-1 receptors when present in their normal location. However, as found with α-MSH, not all cultures were responsive and, as we have previously suggested, we suspect that this was the result of changes at the MC-1 receptor. Nevertheless, it would appear that ACTH peptides can serve as natural ligands of the MC-1 receptor on human melanocytes and their presence in the skin suggests that, together with α-MSH, they may have a role in the regulation of human melanocytes.

Key words: **ACTH, Melanocortin-1 receptor, MSH, Proconvertase, Proopiomelanocortin**

INTRODUCTION

Proopiomelanocortin (POMC) is a 31–36 kDa protein which is enzymatically cleaved to give a number of peptides including the melanocyte-stimulating hormones (MSH), adrenocorticotrophin (ACTH), the lipotrophins (LPH), and the endorphins (Eipper and Mains, 1980). The main site of production of POMC is the pituitary, although it is produced and processed at other sites including the skin. α-MSH was the first of the POMC peptides to be found in the skin (Thody et al., 1983), but since then there have been numerous reports of this and other POMC peptides in the epidermis. Keratinocytes are an important source of these peptides (Slominski et al., 1993; Schauer et al., 1994; Chakraborty et al., 1995; Liu et al., 1995; Wintzen et al., 1996), although they have also been found in melanocytes (Lunec et al., 1990; Farooqui et al., 1993) and Langerhans cells (Morhenn, 1991). Thus, in the skin, POMC

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peptides may function as hormones as well as through paracrine and/or autocrine mechanisms.

The POMC peptides have numerous effects in the skin. For instance, α-MSH, ACTH, and β-LPH have been shown to stimulate sebaceous glands (Thody and Shuster, 1989). It is also well documented that α-MSH and ACTH interact with different cytokines and have immunomodulatory and anti-inflammatory actions (Catania and Lipton, 1993; Bhardwaj and Luger, 1994). The MSH peptides are however best known for their actions on pigment cells and it is well accepted that in lower vertebrates and certain hairy mammals MSH has an important role in the regulation of skin and coat colour, respectively (Thody, 1980; Eberle, 1988). There has been debate concerning the pigmentary significance of the MSH peptides in humans. Since 1961 when Lerner and McGuire first showed that α-MSH produced skin darkening in humans, there have been numerous reports that MSH peptides have no melanogenic actions in cultured human melanocytes (Wilkins et al., 1982; Halaban et al., 1983; Ranson et al., 1988; Friedmann et al., 1990). However, it appears that the lack of response in these earlier studies may have been related to the culture conditions and in more recent studies human melanocytes have been shown to possess functional MSH receptors (Donatien et al., 1992; DeLuca et al., 1993), the activation of which produces an increase in cyclic AMP followed by increases in melanocyte dendricity, tyrosinase activity, and melanogenesis (Thody et al., 1993; Hunt et al., 1994a; Abdel-Malek et al., 1995). The MSH receptor that mediates these effects has been cloned and shown to be a member of a subfamily of the G protein-coupled receptors (Chhajlani and Wikberg 1992; Mountjoy et al., 1992). Several sub-types have been characterized, and one, namely, melanocortin-1 (MC-1) receptor, has been shown to be expressed in melanocytes (Mountjoy et al., 1992).

It is generally supposed that of the naturally occurring POMC peptides, α-MSH is the most active in stimulating pigmentary responses. This may well be true with respect to melanosome dispersing activity in lower vertebrates and the stimulation of melanogenesis in murine melanoma cells (Eberle, 1988). In these assays other POMC peptides such as ACTH1-39 are not particularly active and for this reason ACTH peptides have been dismissed as having significance in the regulation of pigmentation in humans. In a recent study, however, we have observed that ACTH1-39 was equipotent with α-MSH in stimulating melanogenesis in human melanocytes in culture (Hunt et al., 1994b). These two peptides also showed similar binding affinities for the human MC-I receptor following its transfection into COS-7 cells (Chhajlani and Wikberg, 1992). These findings indicate that ACTH1-39 is an agonist of the human MC-1 receptor and since it is present in the circulation at greater concentrations than that of α-MSH (Eberle, 1988), it could serve as an important natural ligand for the MC-1 receptor and consequently, have a role in the regulation of human skin pigmentation. The same may be true of other ACTH peptides that are produced in the skin. In the present study we have therefore characterized the different ACTH peptides that are present in the skin and compared their abilities with those of the MSH peptides in activating the human MC-1 receptor.

MATERIALS AND METHODS

All materials were obtained from Sigma Chemical Co., Ltd (Fancy Road, Poole, Dorset, U.K.) unless otherwise stated.

Skin Samples

These were obtained from children and young adults undergoing surgery and used for the culture of keratinocytes and melanocytes as previously described (Hunt et al., 1994a) and for the immunocytochemical and chromatographic studies.

Cell Culture

Keratinocytes were cultured in MCDB 153 medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 0.18 μg/ml hydrocortisone, 10 μg/ml EGF, and 50 IU/ml penicillin and 50 μg/ml streptomycin (ICN Flow Laboratories, UK) as previously described (Todd et al., 1993). In some experiments the concentrations of Ca^{2+} were increased from 0.07 mM to 1.5 mM in order to induce differentiation. Melanocytes were maintained in serum-free MCDB 153 adjusted to 0.5 mM Ca^{2+} and 0.3 mM tyrosine containing 10 μg/ml insulin, 0.65 ng/ml triiodothyronine, 10 μg/ml transferrin, 0.5 μg/ml hydrocortisone, 50 IU/ml penicillin/50 μg/ml streptomycin, and 50 μg/ml porcine hypothalamic extract prepared from fresh tissue by the method of Macaig et al. (1979), but without phorbol esters, cholera toxin, and other cAMP enhancers (Hunt et al., 1994a).

The human embryonic kidney HEK 293 cells that had been transfected with the human MC-1 receptor were cultured in DMEM supplemented with 10% foetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 250 μg/ml geneticin.

Immunocytochemistry

Cryostat sections of skin that had been air-dried and cultured keratinocytes that had been grown on glass coverslips were fixed in acetone at 4°C for 10 min and immunostained using antibodies directed to ACTH1-14 (DP6), ACTH1-39 (DP4), ACTH18-39 (DP5), and α-MSH (C18-7) and also with antibodies that recognise the prohormone convertases, PC1 and PC2. Further details on the specificity of these antibodies have been described (Estivariz et al.; 1992, Marcinkiewicz et al., 1993, Penny and Thody, 1978). The above antibodies were used at a dilution of 1:1,000, except for PC2, which was used at a dilution of 1:2,000. Antibody reactivity was visualized by the peroxidase reaction by using the Vectorstain kit (Vector Laboratories, UK) and diaminobenzidine (DAB) enhanced with nickel. Primary antibody was omitted from negative controls. Melanocytes and Langerhans cells were identified by their immunostaining with MEL-5 (Signet Laboratories Inc., MA), which detects a 75 kDa pigmentation-associated glycoprotein (Thomson et al., 1985) and T6 (Dako Ltd., High Wycombe, UK), which detects CD1, respectively. MEL-5 was used at a dilution of 1:400 and T6 at 1:200.

Extraction of Peptides

Epidermal samples and cells were placed in 10% acetic acid and stored at -20°C until required. They were then sonicated and the supernatants passed through a Sep-Pak C18 cartridge and freeze-dried. Protease inhibitors (aprotinin 0.01% and phenylmethylsulphonyl fluoride, 1 mM) were added prior to HPLC and radioimmunoassay.

HPLC

A Waters 510 system with a RPC-SC 18 column (4.6 × 270 mm) was used. Linear gradient conditions were established by using solvent A [20% acetonitrile in 0.1% trifuoroacetic (TFA) acid and 0.03% acetic acid]. Samples were injected as 100 µl volumes and separated using a linear gradient from 0–100% solvent B (75% acetonitrile in 0.1% TFA) in 60 min at a flow rate of 0.7 ml/min at 30°C. Two ml fractions were collected for radioimmunoassay. A standard mixture containing the α-MSH peptides, ACTH 1-39, and numerous other ACTH fragments was used to calibrate the system.

Radioimmunoassay

Skin and cell extracts and HPLC fractions were assayed for ACTH and α-MSH using the DP6 and the C18-7 antisera that were used for immunocytochemistry. The DP6 antibody is specific for ACTH1-14 but also detects ACTH1-39 and N-terminal fragments of ACTH (Estivariz et al., 1992). Cross reactivity with α-MSH is less than 0.9%. The C18-7 antibody, on the other hand, is specific for α-MSH peptides and shows negligible cross-reactivity with ACTH1-39 and related fragments (Penny and Thody, 1978). α-MSH and ACTH1-39 were labelled with ^{125}I (IMS 30 Amersham, Buckinghamshire, U.K.) by using Iodogen (1,3,4,6-tetrachloro-3(,6α-diphenyl glycoluril; Pierce, Warriner, Chester,UK) and purified by Sep-Pak or Quso G32, respectively. Bound and free peptide were separated using anti-rabbit antibody-coated cellulose (Sac-cel; IDS Ltd, Bolden, Tyne and Wear, U.K.). All other details were as described previously (Penny and Thody, 1978).

Measurement of Adenylate Cyclase Activity

HEK 293 cells expressing the human MC-1 receptor were washed twice with PBS and then labelled with 37 kBq [^{3}H]-adenine in 1 ml Eagle's medium with 0.1% albumin for 2 hr at 37°C. The radioactive medium was then discarded and the cells washed twice with PBS prior to the addition of medium containing 1 mM isobutylmethylxanthine (IBMX). After 20 min at 37°C, the cells were incubated for a further 45 min in fresh IBMX containing medium with or without the peptides. The reaction was stopped by the addition of ice-cold ethanol. The cells were centrifuged and a sample of the supernatant taken for the measurement of total radioactivity. A nucleotide mixture containing 1 mM cyclic AMP, ATP, ADP, AMP, and adenine to act as a carrier for the labelled cyclic AMP was added to the remaining supernatant, which was then applied to an alumina column (1 × 10 cm). The samples were washed through with 5 mM HCl and the cyclic AMP eluted with 100 mM ammonium acetate. Adenylate cyclase activity was calculated by determining the percentage conversion of [^{3}H]-adenine to [^{3}H]-cyclic AMP.

Effects on Human Melanocytes

Because ACTH1-17 was the most potent of the peptides in activating adenylate cyclase in the above assay, its effect on cultured human melanocytes was examined at concentrations ranging from 10^{-13} to 10^{-8} M. The peptide was added to the cultures and at 24 and 48 hr the melanocytes were examined for morphological changes, i.e., dendricity and at 3 days harvested for the measurement of melanin content as previously described (Hunt et al.,1994a). A total of 13 cultures were set up using melanocytes from fair or brown haired individuals. In cultures where there were insufficient cells for full dose-response curves, the effect of the peptide was examined at concentrations of 10^{-10} and/or 10^{-8} M. Those cultures that showed no increase in dendricity or failed to achieve a 20% or greater increase in their melanin content in response to the peptide were considered to be unresponsive. Whenever possible, i.e., when sufficient melanocytes were available, the effect of 10^{-8} M α-MSH was also examined.

RESULTS

Expression of ACTH and MSH Peptides in the Skin Immunocytochemistry

Immunostaining for ACTH and α-MSH was observed in isolated dermal cells and throughout the epidermis (Fig. 1). No staining was seen in control sections from which the primary antibody had been omitted. Occasional epidermal cells situated suprabasally stained strongly for ACTH and α-MSH. We suspect that these were Langerhans cells, although the numbers of CD1 positive cells were far greater, suggesting that only a minor population of these cells contain POMC peptides. A strong reaction for α-MSH was also seen in cells in the basal layer (Fig. 1b). These showed a similar distribution to cells staining positively with the MEL-5 antibody, suggesting that they were melanocytes. In contrast, staining for ACTH was usually stronger in the upper layers of the epidermis (Fig. 1a), suggesting that keratinocytes show an increased expression of ACTH peptides as they differentiate. This was confirmed in an experiment in which cultured keratinocytes were induced to differentiate. In these keratinocytes immunostaining for ACTH was localised to granules that were dispersed throughout the cytoplasm (Fig. 2).

Immunoreactivity was also detected in the epidermis following the use of antibodies directed to PC1 and PC2. The pattern of staining was, in many respects, similar to that found for the POMC peptides and in the case of PC2 melanocytes often showed a strong reaction (Fig. 3).

Characterisation of ACTH and α-MSH

The presence of ACTH and α-MSH in the skin and cultured keratinocytes was confirmed by radioimmunoassay. As shown in Table 1, the concentrations of ACTH immunoreactivity in the epidermis and in cultured keratinocytes exceeded

Fig. 1. Immunocytochemical localisation of ACTH and α-MSH in human skin. Cryostat sections of normal skin were stained with antibodies to a) ACTH1-14 and b) α-MSH. Strong immunoreactivity can be seen in isolated basal and suprabasal cells. Scale bar, 20 mm.

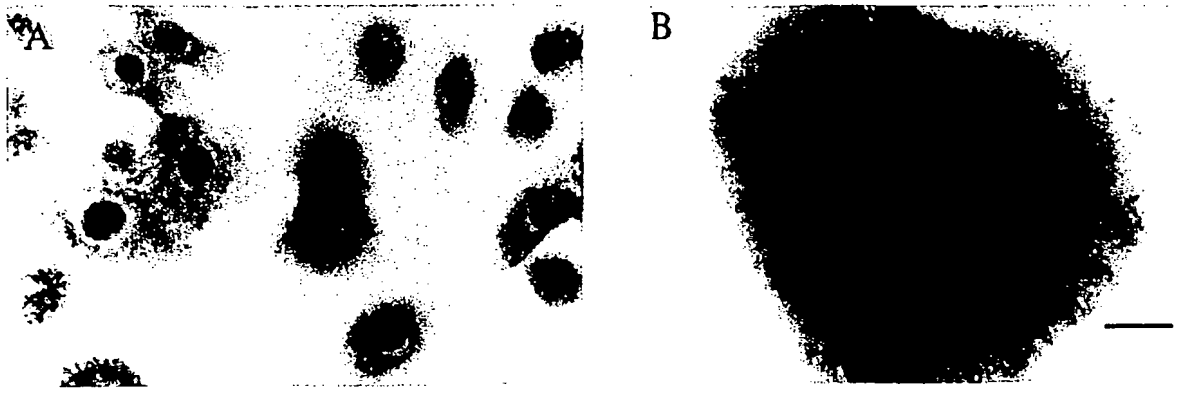

Fig. 2. Immunocytochemical staining of ACTH in human keratinocytes. Keratinocytes were cultured in a) medium containing 0.07 mM Ca2+ and b) in medium containing 1.5 mM Ca^{2+} to induce differentiation. These were then immunostained by using an antibody to ACTH18-39. Note the increased staining in the differentiated cells. Scale bar, 20 mm.

those for α-MSH. HPLC studies revealed several peaks of immunoreactivity, which, on the basis of their retention times and co-elution with synthetic peptides, were identified as ACTH1-39, ACTH1-17, ACTH1-10, acetylated ACTH1-10, α-MSH, and desacetyl α-MSH (Figs. 4, 5). In the epidermis, the majority of the immunoreactive ACTH co-eluted with ACTH1-17 and ACTH1-10, but in the keratinocytes there was proportionally less ACTH1-17 and greater amounts of acetylated ACTH1-10. In the case of α-MSH the elution pattern for the epidermis and the keratinocytes was similar with the major peak co-eluting with desacetyl α-MSH. The small peak that was found in the epidermis and eluted after α-MSH was probably the diacetylated peptide.

Activation of the Human MC-1 Receptor

All peptides examined increased adenylate cyclase activity in HEK 293 cells expressing the human MC-1 receptor (Fig. 6). No increase in activity in response to α-MSH was seen in HEK 293 that lack the MC-1 receptor. The most potent of the peptides was ACTH1-17 with an EC_{50} of 0.43 nM, which compared with 1.08 nM for α-MSH (Table 2). ACTH1-39 was less active with an EC_{50}, which was comparable to that of desacetyl α-MSH.

Effects on Cultured Melanocytes

In the absence of peptides, the melanocytes were mainly bipolar in appearance with an occasional tripolar cell. Within 24 hr of adding ACTH1-17, most of the cells had become dendritic and the effects were usually still evident at 48 hr (Fig. 7). Similar effects were seen with α-MSH (Fig. 7). Such changes were seen in 77% of the cultures in response to ACTH1-17 and in 70% in response to α-MSH (Table 3). Not every culture that responded morphologically responded melanogenically and this was true for both ACTH1-17 and α-MSH. Thus, of the 13 cultures that received ACTH1-17, only 3 showed increases in both melanin and dendricity. These same 3 cultures also showed increases in melanin and dendricity in response to α-MSH (Table 3). The increase in melanin content in response to ACTH1-17 was dose-related and at a concentration of 10^{-9} M was in excess of 200% (Fig. 8). Three of the cultures were totally unresponsive to both ACTH1-17 and α-MSH.

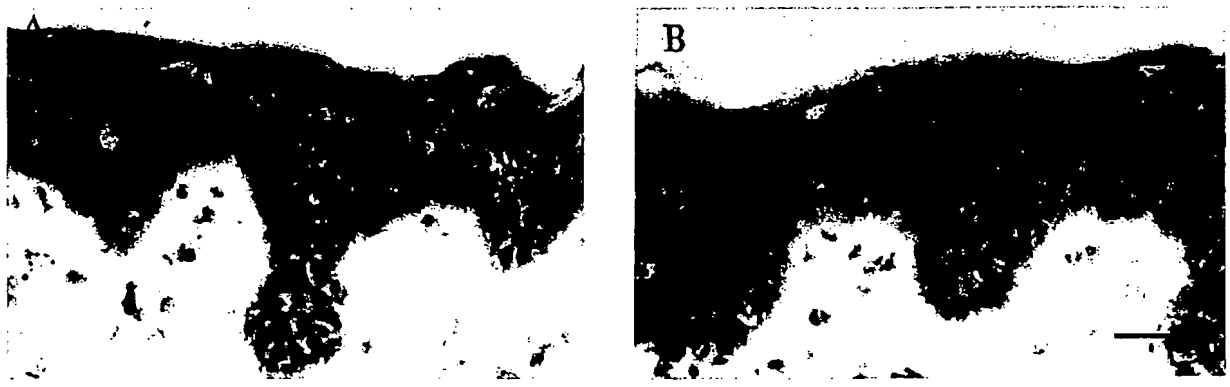

Fig. 3. Immunocytochemical localisation of PC1 and PC2 in human skin. Cryostat sections of normal skin were immunostained using antibodies to a) PC1 and b) PC2. Scale bar, 20 mm.

TABLE I. Concentrations of Immunoreactive ACTH and α-MSH in Human Epidermis and Keratinocytes[a]

Compound	Epidermis (ng/g)	Keratinocytes (pg/10^6 Cells)
ACTH	38.8 ± 2.4	447 ± 35.3
α-MSH	2.73 + 0.09	8.5 ± 0.32

[a]Results are expressed as mean ± S.E.M. of three determinations.

DISCUSSION

In the present study, we have confirmed previous observations that POMC peptides are present in human skin (Thody et al., 1983; Slominski et al., 1993; Liu et al., 1995). By using immunocytochemistry and antibodies specific to α-MSH and ACTH, we observed staining in both the dermis and epidermis. In the epidermis, staining for α-MSH was strong in isolated cells in the basal layer. We suspect that these cells were melanocytes on the basis of their appearance and distribution and the fact that we have previously found the presence of α-MSH in normal and malignant melanocytes (Lunec et al., 1990). In support of this, we have since demonstrated the presence of strong staining for α-MSH in cultured melanocytes (A. Graham and A. J. Thody, unpublished results). It has been reported that Langerhans cells also contain POMC peptides (Morhenn, 1991) and in the present study we observed strong staining, especially for α-MSH, in isolated cells situated suprabasally. These may have been Langerhans cells, but since the numbers were considerably lower than CD1 positive cells, it is possible that only a small proportion of the Langerhans cell population express POMC peptides. In contrast, staining for ACTH was less obvious in isolated cells and appeared to be confined to keratinocytes, especially those in the upper layers of the epidermis, which suggests an increased expression of ACTH as the cells differentiate. This was confirmed in experiments in which cultured keratinocytes were induced to differentiate by increasing the concentration of calcium in the culture medium.

In cultured keratinocytes, immunoreactivity was localized to discrete granules and this is consistent with the view that the peptides are formed as a result of proteolytic cleavage prior to their secretion. The proteolysis of POMC is brought about through the action of proteases known as prohormone convertases, two of which (PC1 and PC2) are present in the pituitary (Seidah et al., 1993). The former, PC1, acts to cleave POMC into ACTH and β-LPH, while PC2 cleaves N-terminally extended ACTH into smaller fragments such as ACTH1-17 and desacetyl α-MSH (Seidah et al., 1993). In the present study, we found evidence of both enzymes in human skin and although the pattern of localization of PC2 tended to parallel that of immunoreactive α-MSH with strong reactions in melanocytes, the co-localization of PC1 and ACTH was less obvious. In fact, staining for PC1 was often strong throughout the basal layer, whereas, as mentioned, basal keratinocytes generally showed a weak reaction for ACTH. The presence of these proteases in the epidermis is also consistent with the recent report that β-LPH and β-endorphin are produced in the skin (Wintzen and Gilchrest, 1996).

In both the epidermis and keratinocytes, the concentration of immunoreactive ACTH was greater than that of α-MSH and this suggests that, although PC2 is present in keratinocytes, ACTH1-39 is cleaved preferentially to smaller ACTH fragments rather than to α-MSH. By using HPLC, we confirmed that the immunoreactive ACTH was composed of several peptides, which, on the basis of their retention times, were identified as ACTH1-17, ACTH1-10, and the acetylated form of ACTH1-10. While processing to such peptides occurs in keratinocytes, the pattern of processing may differ in melanocytes and in these cells it is likely that α-MSH peptides are preferentially produced. This would be consistent with our immunocytochemical findings and our earlier observations that melanocytes contain more α-MSH than keratinocytes (Lunec et al.,1990). It is therefore conceivable that in regulating melanocytes, ACTH peptides are released from keratinocytes and act as paracrine factors, whereas α-MSH peptides may function in an autocrine manner as we have previously proposed (Lunec et al.,1990).

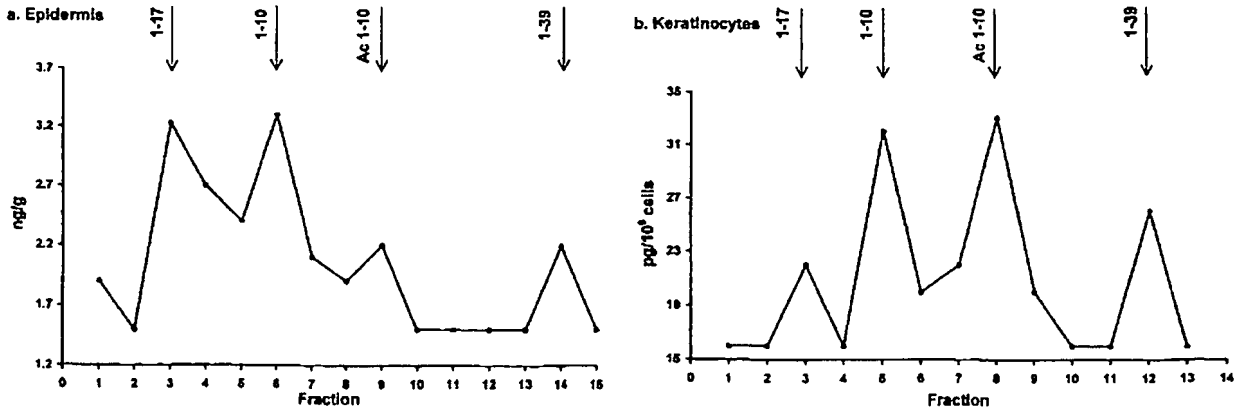

Fig. 4. HPLC analysis of immunoreactive ACTH in extracts of a) epidermis and b) cultured keratinocytes. The amounts of the peptides were measured by radioimmunoassay following HPLC and their identities determined by comparison with the retention times of synthetic peptides.

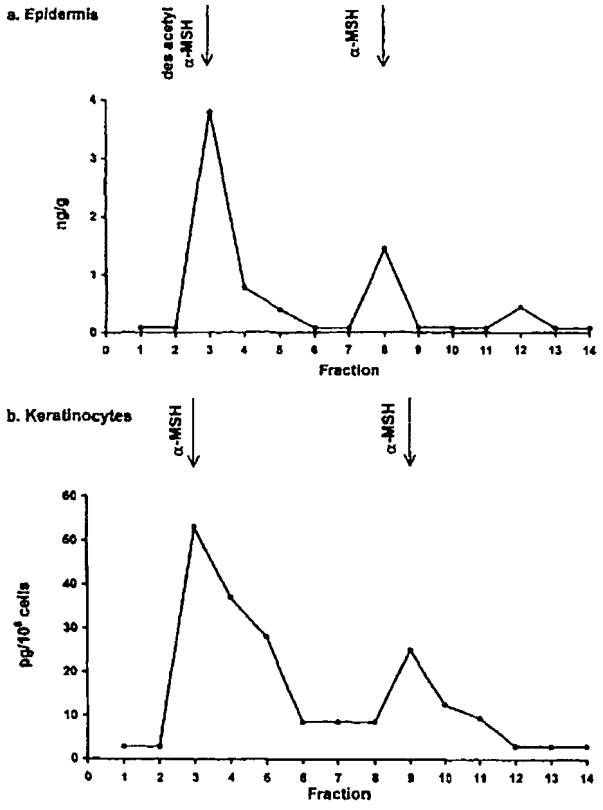

Fig. 5. HPLC analysis of immunoreactive α-MSH in extracts of a) epidermis and b) cultured keratinocytes. The amounts of the peptides were measured by radioimmunoassay following HPLC and their identities determined by comparison with the retention times of synthetic peptides.

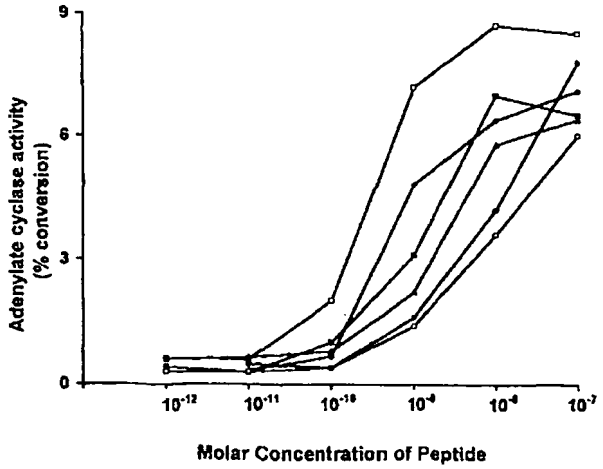

Fig. 6. Activation of the human MC1 receptor by ACTH and α-MSH peptides. Adenylate cyclase activity was measured in HEK293 cells stably transfected with the human MC1 receptor in response to increasing concentrations of the peptides. Open square, ACTH1-17; closed circle, Ac ACTH1-10; closed diamond, α-MSH; closed square, des Ac α-MSH; closed triangle, ACTH1-39; open circle, ACTH1-10. The results are the means of at least three determinations. S.E.M. was around 10% in each case, but error bars have been omitted for clarity.

TABLE 2. Relative Potencies of POMC Peptides at the MC-1 Receptor[a]

Compound	EC_{50} (nM)
α-MSH	1.08 ± 0.21
desacetyl α-MSH	6.56 ± 0.20
ACTH1-39	3.02 ± 1.14
ACTH1-17	0.43 ± 0.06
ACTH1-10	25.0 ± 0.55
ac ACTH1-10	14.1 ± 1.14

[a]Data are the mean ± S.E.M. of at least three experiments. EC_{50} = Concentration of peptide at 50% maximal adenylate cyclase activity. The EC50 of ACTH1-17 was significantly different from that of α-MSH ($P = 0.028$, Mann Whitney non-parametric test).

The presence of acetylated ACTH1-10 suggests that acetylation of the POMC peptides occurred and this was supported by the finding of a peak that co-eluted with α-MSH. There is evidence that in the intermediate lobe of the pituitary POMC peptides undergo N-acetylation prior to their release (Dores et al., 1993). This process takes place in the secretory granule following the proteolytic cleavage reactions and is brought about through the action of N-acetyltransferase. Although ACTH1-39 is a poor substrate the enzyme is capable of acetylating ACTH1-10 (Pease and Dixon, 1981; Glembotski, 1992). The finding of this peptide and acetylated α-MSH in keratinocytes suggests that N-acetylation takes place in these cells as it does in pituitary melanotrophs. This may be important for their secretion and may also have the effect of potentiating their biological actions (Eberle, 1988).

It is generally considered that α-MSH is the most potent pigmentary peptide (Eberle, 1988) and while this may be true in lower vertebrates and mammals such as the mouse, the situation may differ in humans. As we have previously proposed ACTH could be of significance in the regulation of human melanocytes (Hunt et al., 1994b). ACTH is known to be present in human keratinocytes (Schauer et al., 1994; Chakraborty et al., 1995) and as we show here differentiating keratinocytes appear to contain several different ACTH peptides. Although we have previously demonstrated the presence of α-MSH peptides in human epidermis (Thody et al., 1983), this is the first report of different ACTH peptides in human keratinocytes. Experiments were carried out to examine the ability of these ACTH peptides to activate the human MC-1 receptor. To do this we used HEK 293 that had been transfected with human MC-1 and measured the increases in intracellular cyclic AMP in response to the different peptides. All peptides increased adenylate cyclase activity and as expected acetylation increased their potency. Thus, acetylated ACTH1-10 was more potent than the non-acetylated form and the same was true for the α-MSH peptides. Of the peptides tested ACTH1-17 was found to be the most potent. This may reflect increased binding to the receptor since, in an earlier study, it was shown that an analogue of ACTH1-17 binds to the MSH receptor on a human melanoma cell line with an affinity that was 22-fold higher than that of α-MSH (Siegrist et al., 1989). As the present results show ACTH1-17 stimulated dendricity and melanogenesis in cultured hu-

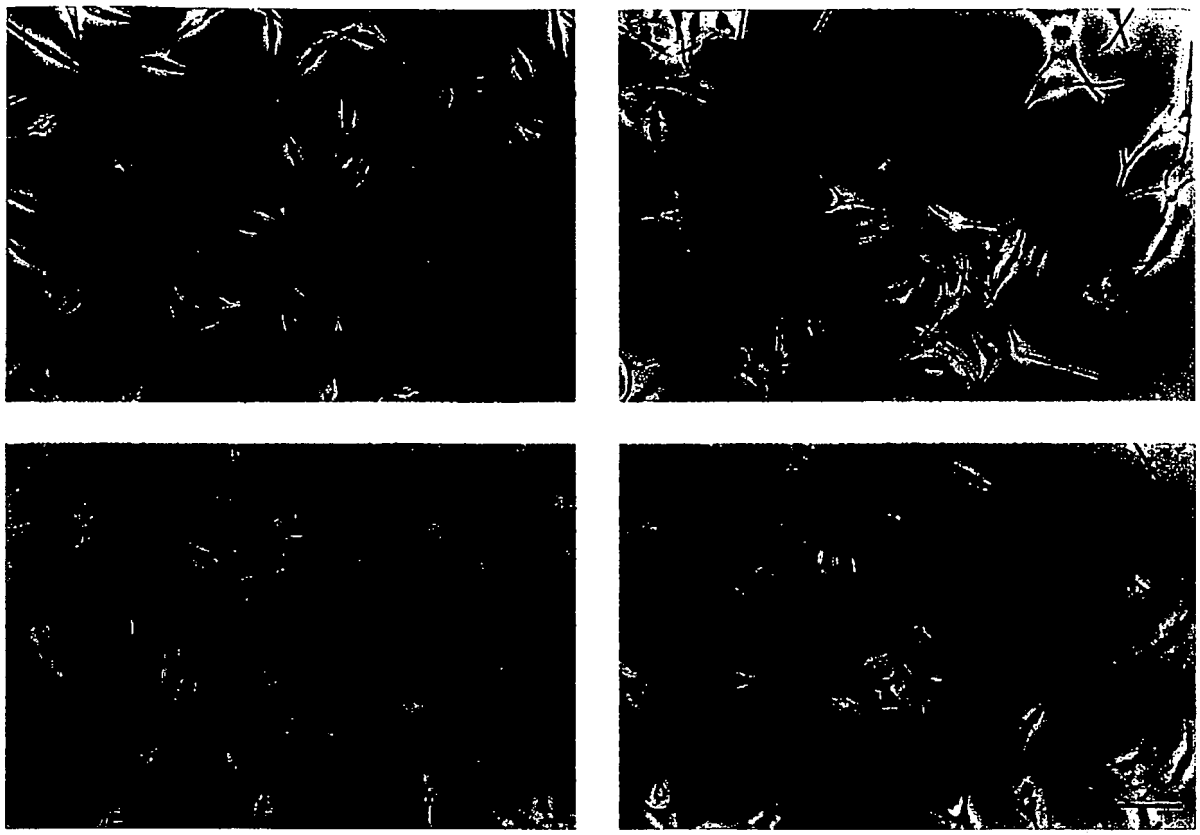

Fig. 7. The effects of ACTH1-17 on the morphology of cultured human melanocytes. Melanocytes (2×10^5) were seeded into the wells of a six-well plate and left to attach overnight in 2 ml MCDB 153. ACTH1-17 was then added and the cells reincubated for up to 48 hr. Effects are compared with those of α-MSH. a) Untreated control, b) α-MSH (10^{-8} M) for 24 hr, c) ACTH1-17 (10^{-8} M) for 24 hr, d) ACTH1-17 (10^{-8} M) for 48 hr. Scale bar, 20 mm.

man melanocytes confirming that the peptide is capable of activating the MC-1 receptor when present in its normal location on the melanocyte. However, not all cultures responded to the peptide and these same cultures were also unresponsive to α-MSH. We have previously reported that some melanocytes fail to respond to α-MSH and ACTH1-39 (Hunt et al., 1994a,c, 1996) and we suspect that this is related to changes at the MC-1 receptor. We have found a high frequency of MC-1 receptor variants in individuals with red hair and/or fair skin (Valverde et al., 1995) and melanocytes from such phenotypes are unresponsive to α-MSH (Hunt et al., 1996). In the present study the melanocytes were obtained from fair or brown-haired Europeans and on the basis of our earlier findings we would predict that a small percentage would be unresponsive to agonists of the MC-1 receptor. On the basis of the present findings, we would also predict that in individuals with functional MC-1 receptors ACTH1-17 may be just as important as a ligand at this receptor as α-MSH. The possibility should therefore be considered that ACTH peptides that are present in the skin may act together with α-MSH in regulating melanocyte function.

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TABLE 3. Responsiveness of Melanocyte Cultures to ACTH1-17 and α-MSH[a]

Peptide	No. of cultures	Responsive			Non-responsive
		↑dendricity+ ↑melanin	↑dendricity	↑melanin	
ACTH1-17	13	3 (23)	7 (54)	0	3 (23)
α-MSH	10	3 (30)	3 (30)	1 (10)	3 (30)

[a]Results are given as numbers of responsive and non-responsive cultures with percentages in parenthesis. Each culture comprises melanocytes from a single individual.

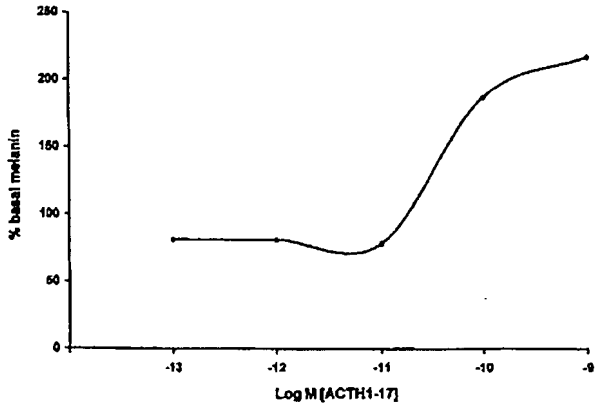

Fig. 8. Effect of ACTH1-17 on melanin content of cultured human melanocytes. A typical dose-response curve from a responsive culture is shown. Data are expressed as % basal melanin.

bodies to PC1 and PC2, and Dr. W.J. Chen and Dr. W.O. Wilkison for the transfected HEK 293 cells.

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Original Article

Expression of the corticotropin-releasing hormone–proopiomelanocortin axis in the various clinical types of psoriasis

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Abstract: Psychological stress is known to aggravate inflammatory skin diseases such as atopic dermatitis, psoriasis and contact sensitivity by altering the cellular constituents of the immune system. The skin appendages function dually as prominent targets and sources of the peripheral corticotropin-releasing hormone–proopiomelanocortin (CRH-POMC) axis. In this study, we examined the expression level of CRH-POMC axis constituents in psoriasis, a well-known stress-related inflammatory skin disease. The 15 psoriasis patients and six normal controls were retrospectively selected after extensive review of their clinical records and skin biopsy specimens. We immunohistochemically analysed the expressivity of CRH, adrenocorticotrophic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) in various types of psoriatic lesions and control skin. A significant increase of CRH expression was observed in psoriatic lesions, which involved the entire epidermis (upper layer in particular), hair follicles and sweat glands compared with controls. Expression of ACTH and α-MSH was clearly stimulated in a subset of psoriasis patients compared with controls, but on the whole, lacked statistical significance. The immunoreactivity of CRH, ACTH and α-MSH in psoriasis was not dependent on its clinical subtype, duration or number of previous treatments. Compared with the definite increase of CRH expression in psoriasis, the expression of the POMC peptides was heterogenous with no overall significance. From the findings, we suggest that CRH, a key stress hormone, may play an important role in the pathomechanism of psoriasis.

Key words: CRH–POMC axis – psoriasis – stress

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Introduction

Corticotrophin-releasing hormone (CRH) is a 41-amino acid peptide synthesized not only inside the hypothalamus upon activation of the hypothalamic–pituitary–adrenal (HPA) axis (1,2), but is also produced outside the hypothalamus. CRH and CRH receptors (CRHRs) have been identified in peripheral tissues, which include the adrenal glands, gonads, placenta, gastrointestinal system, pancreas and the skin (3,4). Cutaneous CRH is believed to regulate various functions of the skin, although its exact mechanism is unknown (4–8). Normal human skin expresses the proopiomelanocortin (POMC) gene and contains transcripts of both CRH and CRHRs. In fact, an equivalent of the central HPA axis is activated in the skin in response to stress (3,4,8). Therefore, it is natural for us to consider the skin as a target organ and major source of CRH and POMC peptides at the same time (3).

The stress response is subserved by the central nervous system and periphery. Their principle effectors include CRH, adrenocorticotrophic hormone (ACTH), POMC peptides, glucocorticoids and cathecolamines such as noradrenaline and adrenaline (9). Activation of the HPA axis and sympathetic nervous system are normal following acute

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotrophic hormone; AEC, 3-amino-9-ethylcarbazole; CRH-POMC axis, corticotrophin-releasing hormone–proopiomelanocortin; CRHR, CRH receptors; POMC, proopiomelanocortin; VEGF, vascular endothelial growth factor.

*These authors contributed equally to this work.

psychic stress. However, patients with psoriasis have shown blunted cortisol response to acute stress (10). Such inappropriate responsiveness of the stress system may account for a number of endocrinologic, immunologic and psychiatric disorders (9).

Psychological stress may trigger psoriasis. Many have suggested activation of the central and peripheral HPA axis with subsequent increase in stress hormone levels most likely to cause the exacerbation (11–15), but with little supportive data. In this immunohistochemical study, we compared the expression level of CRH, ACTH and α-MSH in psoriatic lesions with that in normal control in order to determine their role in psoriasis.

Patients, materials and methods

Patients and skin biopsy specimens

In this retrospective study, 15 patients with skin-biopsy-confirmed psoriasis were selected from our outpatient clinic. Control skin specimens were obtained from the six patients who underwent cosmetic surgery. The study was approved by the ethical committee of the Catholic University of Korea and all patients filed in an informed consent. In the psoriasis group, the involved area, clinical subtype, duration of illness, and modes of previous therapy were examined, which is well-summarized in Table 1.

The paraffin blocks were collected, cut in 5-μm-thick sections, dried at 37°C, deparaffinized in xylene and rehydrated in a graded series of ethanol before use. For standard histology, the specimens were fixed in 4% formalin and processed for haematoxylin and eosin staining.

Immunohistochemistry

We detected CRH, ACTH and α-MSH peptide expression in tissue sections using their respective rabbit polyclonal antibodies (Phoenix Pharmaceutical, Inc., Belmont, CA, USA). The antibodies were diluted in phosphate-buffered saline plus 0.1% Triton X-100 at a ratio of 1:500 and incubated overnight at 4°C in a wet chamber. We subsequently added the biotinylated secondary antibody and peroxidase-conjugated streptavidin complex (Zymed Laboratories Inc., San Francisco, CA, USA) to the mixture. 3-Amino-9-ethylcarbazole (AEC) was used as a chromogenic substrate. Normal rabbit IgG (Zymed Laboratories Inc.) was used for negative control. CRH, ACTH and α-MSH expression was graded as the percentage of positive staining cells: 0 for negative, + for 1–33% positive, ++ for 34–66% positive and +++ for 67–100% positive. Two independent dermatopathologists reviewed the results. Fisher's exact test was adopted for statistics where $P < 0.05$ was considered significant.

Results

Enhanced expression of CRH peptides in psoriasis

In the control group, three samples did not stain at all, but the remaining stained + in the hair follicles (one case) and sweat glands (three cases) (Fig. 1, Table 2). In contrast, all 15 specimens from the psoriasis group stained positive in the epidermis, hair follicles and sweat glands for CRH (Fig. 1, Table 2). It is noteworthy that 87% of the psoriatic epidermis stained strong (++/+++) for CRH (Fig. 1, Table 2). Compared with normal control, CRH expression in the epidermis, hair follicles and sweat glands were signi-

Table 1. Characteristics of psoriasis patients

Patent	Sex	Age	Area involved	Type[1]	Duration	Therapy
1	M	3	Back	Large plaque	3 days	Topical treatment
2	M	29	Arm	Small plaque	1 year	UVB, topical treatment
3	F	56	Trunk	Small plaque	5 years	Etretinate, cyclosporine
4	F	36	Arm	Small plaque	15 days	Etretinate
5	F	61	Arm, buttock	Small plaque	1 month	Topical treatment
6	M	16	Trunk	Large plaque	1 year	UVB, topical treatment
7	F	28	Trunk	Small plaque	3 months	UVB
8	M	40	Thigh	Small plaque	4–5 years	Topical treatment
9	F	37	Scalp, trunk	Guttate	1 week	Topical treatment
10	M	28	Trunk	Small plaque	6 years	Etretinate
11	F	46	Trunk	Guttate	2 weeks	Acitretin, topical treatment
12	F	27	Trunk	Small plaque	4 months	UVB, topical treatment
13	F	13	Knee, elbow	Small plaque	1 year	Topical treatment
14	M	50	Whole body	Small plaque	20 years	Topical treatment
15	F	24	Knee, axilla, hand	Small plaque	14 years	Topical treatment

[1]Fitzpatrick's Dermatology in General Medicine (6th edition).

ficantly increased in the psoriasis group ($P < 0.05$) (Table 2). Although CRH intensity in the dermis was not scored, we identified few samples of the psoriasis group with immunoreactive cells in the dermis (Fig. 2).

Expression of ACTH and α-MSH peptides in psoriasis

While there was only weak (0 to +) expression of POMC peptides in the control group, few strong (++ to +++) expressions were observed in the psoriasis group (Fig. 1). One of the control samples stained + for ACTH (epidermis, hair follicles and sweat glands) and α-MSH (epidermis and sweat glands), but staining was absent in the remaining cases (Table 2). Of the 15 patients in the psoriasis group, + to ++ staining for ACTH (epidermis: seven of 15 cases, hair follicles: five of eight, sweat glands: four of 15) and α-MSH (epidermis: nine of 15 cases, hair follicles: two of eight, sweat glands: two of 15) were seen, respectively, but neither ACTH nor α-MSH stained +++ (Table 2). In the psoriasis group, the intensity of ACTH and α-MSH seemed stronger in the suprabasal layer compared with the lower half of the epidermis (Fig. 2).

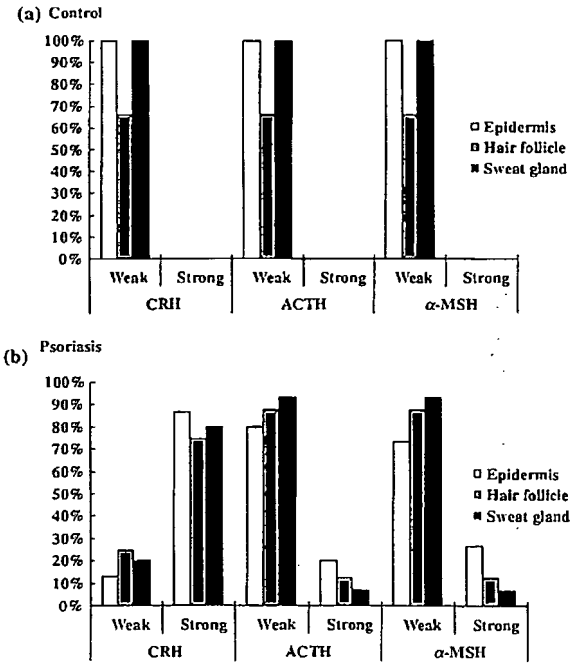

Figure 1. Immunoreactivity of CRH, ACTH, and α-MSH in the control and psoriasis group graded either weak (0 and +) or strong (++ and +++). Grading was based on the percentage of positive staining: + for 1–33%, ++ for 34–66% and +++ for 67–100%. (a) CRH and POMC peptides are only weakly expressed in controls ($n = 6$). (b) Strong positive staining of CRH is detected in 87% of psoriasis patients, but the expression of POMC peptide is variable with a small proportion showing strong reactivity.

Discussion

Corticotropin-releasing hormone is a major regulator of the HPA axis in response to stress. CRH stimulates the CRHR on the pituitary corticotrophs, which in turn, induces the production and secretion of POMC peptides such as ACTH, melanotropins, lipotropins and endorphins (1–4). The existence of a local stress response system equivalent to the central HPA axis has been demonstrated

Table 2. Immunoreactivity of CRH, ACTH and α-MSH in normal control and psoriasis patients

	Intensity of immunostaining					
	0	+	++	+++	Total	*P*-value
CRH						
Epidermis						
Normal	6	0	0	0	6	**<0.01**
Psoriasis	0	2	5	8	15	
Hair follicle						
Normal	3	1	0	0	4[1]	**<0.05**
Psoriasis	0	2	2	4	8[1]	
Gland						
Normal	3	3	0	0	6	**<0.01**
Psoriasis	0	3	5	7	15	
ACTH						
Epidermis						
Normal	5	1	0	0	6	>0.05
Psoriasis	8	4	3	0	15	
Hair follicle						
Normal	3	1	0	0	4[1]	>0.05
Psoriasis	3	4	1	0	8[1]	
Gland						
Normal	5	1	0	0	6	>0.05
Psoriasis	11	3	1	0	15	
α-MSH						
Epidermis						
Normal	5	1	0	0	6	>0.05
Psoriasis	6	5	4	0	15	
Hair follicle						
Normal	4	0	0	0	4[1]	>0.05
Psoriasis	6	1	1	0	8[1]	
Gland						
Normal	5	1	0	0	6	>0.05
Psoriasis	13	1	1	0	15	

The signal was graded as the percentages of positive staining cells: 0 for negative, + for 1–33%, ++ for 34–66% and +++ for 67–100%. Two dermatopathologists reviewed the results independently. The chi-square test was performed for statistical significance.
[1]The sample size is <6 in the control group and <15 in the psoriasis group because a number of samples did not show any hair follicles.

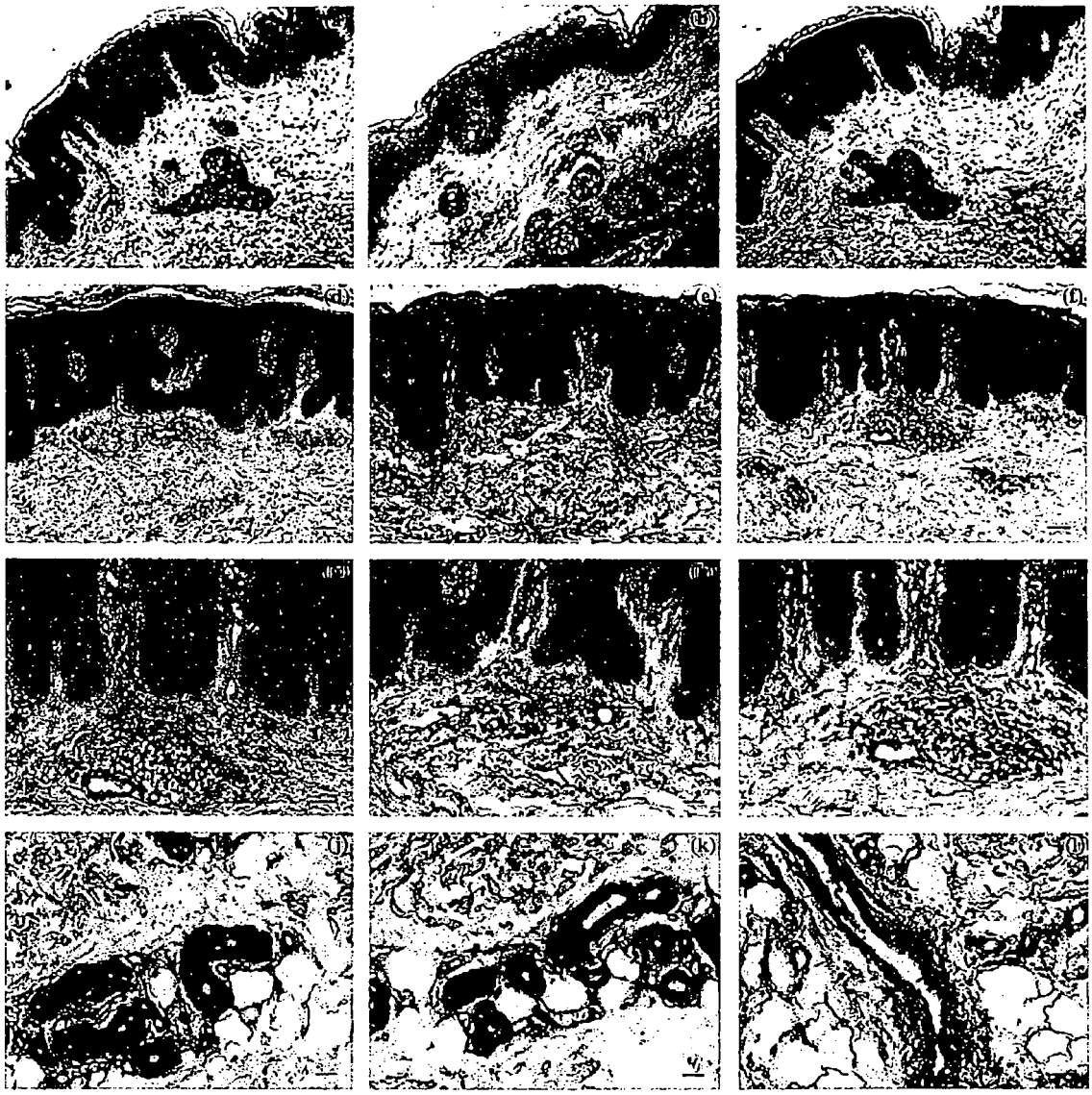

Figure 2. Immunohistochemical staining of CRH, ACTH and α-MSH in normal control and psoriasis group. CRH, ACTH and α-MSH is only weakly stained in normal control (a–c). In the psoriasis group, CRH expression is increased in the epidermis (particularly the upper layer) (d), the perivascular infiltrating cells (g), and sweat glands (j). Weak immunoreactivity of ACTH and α-MSH is observed in the upper layers of the epidermis (e, f), dermal infiltrating cells (h, i), and sweat glands (k, l), respectively. *Immunoreactive areas in the epidermis and sweat glands. Arrows point the immunopositive perivascular infiltrating cells. Scale bar: (a–f) 50 μm, (g–l) 25 μm, respectively.

(3,4,6,8,16). Paus et al. (17) suggested the skin as an ultimate model for neuroimmunological stress research. In this study, we examined the role of CRH, ACTH and α-MSH in psoriasis by grading their respective immunoreactivity and tried to differentiate the clinical subtypes of psoriasis by these expressions. In the psoriasis group, all cases expressed CRH peptides in the epidermis, hair follicles and sweat glands of which the intensity was significantly elevated compared with controls. In contrast, while the expression of ACTH and α-MSH peptides were clearly increased in a small subset of patients with psoriasis, it was totally absent in the remaining population, indicating a heterogeneous response. We think that the response merely presents interpersonal variance, but further study on this subject should be made. Overall, the findings are consistent with previous reports on skin tumor and inflammatory skin dis-

ease such as alopecia areata, where upregulation of CRH and POMC peptides have been detected (18–20). Kono et al. (11) have detected CRH and POMC expression in psoriatic lesions but its statistical significance could not be identified with the lack of grading. So far, no report has compared the expression of CRH and POMC peptides in the different subtypes of psoriasis. To our knowledge, this is the first study to compare the expression of CRH and POMC peptides in the guttate, small plaque and large plaque form of psoriasis, which we consider meaningful.

As shown in Table 1, various clinical subtypes were identified in the psoriasis group. Two patients had a guttate type with an acute onset. Eleven patients had a small plaque type and two presented with a large plaque type of psoriasis. The duration of the illness varied from 3 days to 20 years. Most patients had been treated with topical or systemic therapy including phototherapy. The increase in CRH immunoreactivity was similar in all psoriatic lesions regardless of the type of psoriasis, chronicity of disease and previous treatment, which indicates the role of CRH in the pathogenesis of psoriasis. At present, its action mechanism is unclear but the proinflammatory effect of CRH on keratinocytes (21–23) or activation of mast cells is highly suspected (24–26). Recently CRH has been shown to stimulate production of interleukin (IL)-6, IL-11 in human HaCaT keratinocytes during cutaneous stress (23). Psoriasis lesions contain a larger number of mast cells which have a higher CRH-R and CRH expression level (24,25). CRH may activate mast cells via a CRH-R-dependent mechanism, leading to release of histamine and hence vasodilatation with increased vascular permeability (26). Mast cells secrete numerous proinflammatory cytokines such as IL-6, IL-8 and tumor necrosis factor-α, which are released in response to stressful stimuli (25). They are recognized as potent stimulators of CRH and POMC production in human skin (6). CRH also modulates the expression of cell adhesion molecules like intercellular adhesion molecule-1, which promotes the proliferation of lymphocyte, and enhances the vascular permeability via increased vascular endothelial growth factor (VEGF) (25). Interestingly, it was reported that transgenic delivery of VEGF to mouse skin led to an inflammatory condition that resembled human psoriasis (27).

In summary, CRH expression was significantly elevated in the epidermis and skin appendages of psoriatic lesions without any subtype-dependent variations. The relative blunt expression of POMC peptides (malfunctioning of the executive arm of the HPA axis) in psoriasis may account for the overall inflammation as proinflammatory actions of CRH are not sufficiently counteracted by the action of POMC peptides (14,28–30). From our study, we identified CRH as a crucial factor in the pathogenesis of psoriasis, but its action mechanism should be further clarified with additional research.

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Exhibit E

Peptides, Vol. 5, pp. 841–843, 1984. © Ankho International Inc. Printed in the U.S.A. 0196-9781/84 $3.00 + .00

BRIEF COMMUNICATION

Regional Heterogeneity in the Ratio of α-MSH:β-Endorphin in Rat Brain

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MILLINGTON, W. R., G. P. MUELLER AND T. L. O'DONOHUE. *Regional heterogeneity in the ratio of α-MSH: β-endorphin in rat brain.* PEPTIDES 5(4) 841–843, 1984.—The molar ratio of α-MSH:β-endorphin varies markedly among discrete microdissected regions of rat brain ranging from 0.57 in the median eminence to 2.74 in the lateral septum. This finding demonstrates that α-MSH and β-endorphin (β-END) are not uniformly distributed in a 1:1 molar ratio in rat brain as one might predict based on the consideration that the two peptides are synthesized in equimolar amounts as part of a common precursor molecule, pro-opiomelanocortin. The data indicate instead that the concentrations of α-MSH and β-END, the two predominant peptides expressed by opiomelantropinergic neurons, are independently regulated in rat brain. The heterogeneity of α-MSH:β-END ratios suggests that the regulation of α-MSH and β-END is regionally specific and may impart functional selectivity to the multisecretory opiomelanotropinergic neuronal system.

β-Endorphin α-Melanocyte stimulating hormone α-MSH Neuropeptide co-localization

OPIOMELANOTROPINERGIC neurons are prominent members of a rapidly expanding class of chemically defined neuronal systems that co-synthesize multiple neuropeptide transmitters from larger precursor molecules. α-MSH and β-endorphin (β-END), the predominant neuropeptides expressed by the opiomelanotropinergic neuronal system [2,7], coexist within the processes of an extensive fiber distribution which emanates from a single group of perikarya localized in the medial basal hypothalamus and projects to subcortical structures throughout the rat forebrain [3, 16, 18]. The opiomelanotropinergic neuronal system is thought to participate in the integration of a variety of apparently diverse behavioral, endocrine and autonomic functions [1, 4, 11, 15]. It is far from certain, however, exactly how the expression of multiple peptide transmitters contributes to the regulation of these physiologic processes. Here we report that the relative concentrations of immunoreactive α-MSH (iα-MSH) and iβ-END, the iα-MSH:iβ-END ratio, varies markedly among discrete microdissected regions of rat brain. The regional heterogeneity of peptide ratios suggests that presynaptic regulatory mechanisms independently control iα-MSH and iβ-END levels and may thereby impart functional selectivity to specific neuronal pathways within the opiomelantropinergic neuronal system.

METHOD

Male Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) (200–250 g) used in these studies were housed six to a cage with free access to food and water under a twelve hour light:dark cycle. The rats were killed by decapitation between 10.00 and 12.00 hours, the brains were rapidly removed, mounted on a microtome chuck and frozen on dry ice. Thick sections (300 μm) were cut on a cryostat and microdissected using a technique originally described by Palkovits [17]. The sectioning coordinates and the number and size of the individual microdissected regions have been described previously [16].

Microdissected regions from two animals were combined and homogenized by sonication in 200 μl of 1 N acetic acid. A 4-20 μl aliquot was removed from each sample for protein determination [13] and the samples were centrifuged at 8,000 × g and 4°C for thirty minutes. Aliquots (40 μl) of each supernatant fluid were removed, lyophilized and total iα-MSH and iβ-END were analyzed in duplicate by radioimmunoassay.

iβ-END was analyzed using an antiserum directed against the C-terminal portion of camel β-END (β-END_{16}-β-END_{25}) [14]. The antiserum crossreacts on an equal molar basis with β-lipotropin (β-LPH) with both the free and N-acetylated forms of β-END_{1-31} and β-END_{1-27} but exhibits less than 1% crossreactivity with ACTH, α-MSH, β-MSH, α-endorphin and methionine and leucine enkephalin. The detection limit of the assay is less than ten picograms of iβ-END at a final antiserum dilution of 1:125,000. The α-MSH antiserum was

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TABLE 1

THE DISTRIBUTION OF α-MSH AND β-END CONCENTRATIONS AND α-MSH:β-END RATIOS IN RAT BRAIN

Region	Concentration (fmoles/μg protein) α-MSH	β-END	Ratio α-MSH:β-END
Lateral Septum	0.38 ± 0.14	0.12 ± 0.02	2.74 ± 0.24*
Interstitial nucleus of the Stria Terminalis	2.20 ± 0.38	1.88 ± 0.20	1.18 ± 0.03
Medial Preoptic n.	2.76 ± 0.40	1.64 ± 0.21	1.72 ± 0.09*
Anterior Hypothalamic n.	2.36 ± 0.37	1.63 ± 0.12	1.32 ± 0.09
Periventricular n.	3.76 ± 0.28	3.34 ± 0.26	1.16 ± 0.14
Supraoptic n.	1.04 ± 0.13	0.61 ± 0.17	1.76 ± 0.31*
Paraventricular n.	1.87 ± 0.20	2.41 ± 0.31	0.81 ± 0.08
Arcuate n.	5.10 ± 0.64	6.56 ± 1.16	0.85 ± 0.20
Median Eminence	2.98 ± 0.19	7.61 ± 2.24	0.57 ± 0.16
Ventromedial n.	2.15 ± 0.19	1.54 ± 0.12	1.27 ± 0.05
Dorsomedial n.	2.87 ± 0.56	2.93 ± 0.23	1.06 ± 0.10
Posterior Hypothalamic n.	1.49 ± 0.28	0.74 ± 0.22	1.85 ± 0.37*
Periventricular n. of the Thalamus	2.72 ± 0.36	4.44 ± 1.09	0.68 ± 0.14
Medial Amygdaloid n.	0.43 ± 0.04	0.20 ± 0.02	2.18 ± 0.17*
Central Gray, Rostral	0.49 ± 0.04	0.35 ± 0.06	1.49 ± 0.24*
Central Grey, Caudal	1.14 ± 0.04	1.16 ± 0.01	0.98 ± 0.02

*p less than 0.05 differs from the arcuate nucleus.

Brain regions from ten rats were microdissected using the Palkovits technique [15]. Individual samples from two animals were combined and assayed for α-MSH and β-END immunoreactivities by radioimmunoassay. Each value represents the mean ± SEM of 4-5 determinations. The data were analyzed by analysis of variance followed by Duncans multiple range test.

raised against synthetic α-MSH [16]. It crossreacts equally with α-MSH, desacetyl-α-MSH, N,O diacetyl-α-MSH and with the methionine sulfoxide derivatives of all α-MSH related peptides. The antiserum exhibits 0.3% crossreactivity with β-MSH and less than 0.1% crossreactivity with ACTH, β-LPH and all known forms of β-END. The α-MSH antiserum binds 30–35% of trace ^{125}I-α-MSH at a final dilution of 1:50,000 and the assay has a limit sensitivity of five picograms. The intra- and inter-assay coefficients of variation of both radioimmunoassays are less than five and ten percent respectively.

RESULTS AND DISCUSSION

The molar ratio of total immunoreactive α-MSH:β-END varies over a five-fold range among discrete microdissected regions of rat brain (Table 1) (ANOVA $F(14,51)=12.12$, $p<0.001$, n=4–5). The concentration of iα-MSH is highest relative to iβ-END levels in the lateral septum (2.74±0.24) and lowest in the median eminence (0.57±0.16) and in the periventricular nucleus of the thalamus (0.68±0.14). Significant differences occur between the iα-MSH:iβ-END ratio in the arcuate nucleus, a region containing opiomelanotropinergic neuronal perikarya, and the peptide ratios in the septum, preoptic nucleus, posterior hypothalamus, medial amygdala and the rostral central gray ($p<0.05$). Thus, the relative concentrations of iα-MSH and iβ-END occurring in individual brain regions are not well correlated either with the distance separating the region from the arcuate nucleus or with the density of innervation of that region by opiomelanotropinergic neuronal processes. These data indicate that iα-MSH and iβ-END are not uniformly distributed in a 1:1 molar ratio as one might predict based on the consideration that both peptides are synthesized in equimolar amounts as part of a larger precursor molecule pro-opiomelanocortin (POMC) [5,12]. The results suggest instead that iα-MSH and iβ-END levels are independently regulated either by differential post-translational processing of their immediate precursors, ACTH and β-LPH, or because the turnover rates of the two peptides differ among different terminal projection fields of opiomelanotropinergic neurons.

The results of many previous investigations indicate that iα-MSH and iβ-END peptides coexist in rat forebrain within the processes of a single neuronal population whose perikarya are localized exclusively to the arcuate nuclues and adjacent regions of the medial basal hypothalamus (MBH) [1, 3, 15, 16, 18]. Recently a second group of POMC containing cell bodies has been identified in the nucleus tractus solitarius (nTS) [10,19] and in the lateral reticular formation [19] of rat brainstem. This raises the possibility that regional variations in the ratio of iα-MSH:iβ-END may result from the differential distribution of rostral projections from the nTS to forebrain structures. The contribution of the nTS cell group to forebrain iα-MSH and iβ-END concentrations does not appear to be significant, however, because lesioning the arcuate nucleus lowers forebrain opiomelanotropin peptide levels by ninety percent or more [6,16]. α-MSH immunoreactive perikarya have also been identified in the dorso-lateral hypothalamus [8,20]; these cells predominantly innervate the cortex, hippocampus and caudate nucleus [9]. This neuronal system is labeled immunohistochemically by some (but not all [3,16]) α-MSH antisera but is not

stained by antisera to POMC or any other POMC-derived peptide suggesting that the immunoreactive material may be a peptide structurally related to α-MSH rather than α-MSH itself. The possibility that this cell group contributes to the regional heterogeneity of iα-MSH:iβ-END ratios is particularly unlikely, however, because the α-MSH antisera used in these studies does not recognize α-MSH immunoreactive perikarya in the dorso-lateral hypothalamus [16]. That arcuate nucleus lesions reduce the α-MSH content of the dorsolateral hypothalamus to the same extent as other subcortical regions (unpublished observations) further demonstrates that all of the α-MSH detected immunochemically in these experiments originates from the MBH. Thus the occurrence of regional variations in the distribution of iα-MSH:iβ-END ratios indicates that the MBH opiomelanotropinergic neuronal system is not homogeneous. Opiomelanotropinergic neuronal processes contain and presumably release different relative amounts of iα-MSH and iβ-END, the two predominant POMC-derived peptides, in different terminal projection fields of the opiomelanotropinergic neuronal system.

The neuronal mechanisms responsible for regional differences in the expression of iα-MSH:iβ-END ratios remain to be determined. The explanation may lie in the anatomical connectivity of the opiomelanotropinergic neuronal system; it is possible that separate neuronal populations containing different peptide ratios project to different terminal field regions. On the other hand, our data are also consistent with the concept that iα-MSH:iβ-END ratios vary among different axon collaterals of individual neurons. This hypothesis suggests that the relative amounts of iα-MSH and iβ-END released from different processes of individual neurons may vary depending on the synaptic organization of specific brain nuclei. In either case the hetereogeneity of iα-MSH:iβ-END ratios suggest that the expression of opiomelanotropin peptides is regionally selective and may impart functional specificity to the multisecretory opiomelanotropinergic neuronal system.

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measurement

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Quantification of the Permeability of the Blood-CSF Barrier to α-MSH in the Rat

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WILSON, J. F., S. ANDERSON, G. SNOOK AND K. D. LLEWELLYN. *Quantification of the permeability of the blood-CSF barrier to α-MSH in the rat.* PEPTIDES 5(4) 681–685, 1984.—The ability of α-MSH to cross the blood-CSF barrier of the rat was assessed by measurement of the rate of appearance of immunoreactive α-MSH in a cerebrospinal fluid (CSF) perfusate following intravenous injection of peptide. Comparisons were made with the rate of appearance of a simultaneously administered dose of ^{14}C-inulin which is poorly permeable at the blood-CSF barrier. Concentrations of drugs measured in plasma were fitted to two-compartment pharmacokinetic models, and those measured in the CSF perfusate to one-compartment open systems receiving an input from the plasma compartment. The rate constant for entry of α-MSH into CSF was 0.00087 min^{-1}, which was not significantly different from that for inulin of 0.00055 min^{-1}. As α-MSH penetrated into CSF at a rate comparable to inulin, it was concluded that the limited entry of peptide was by aqueous diffusion along with other water-soluble macromolecules.

α-MSH Inulin Blood-CSF barrier Cerebrospinal fluid Rat

α-MELANOPHORE-stimulating hormone (α-MSH) is one of a family of peptides originating from the pro-opiocortin precursor. It is known to be produced both in the pars intermedia of the pituitary gland and in neurones of the arcuate nucleus of the hypothalamus, and to circulate both in blood and in cerebrospinal fluid (CSF) [4, 17, 23]. Peripheral administration of the peptide results in centrally mediated effects, notably on learning and memory [5, 10, 17]. The possible role of blood-borne peptide in central nervous system (CNS) function and the route by which such effects could be mediated are thus of interest.

Penetration of solutes directly into the brain is restricted by the blood-brain barrier (BBB) and the blood-CSF barrier which impede the passage of relatively non-lipid-soluble molecules of molecular weight greater than 500 such as most peptides [13,18]. Alternative routes of entry involve carrier-mediated transport systems or the circumventricular organs where the BBB is absent. To exert central effects, however, peptides need not accumulate generally within the brain [10,13]. For example, α-MSH has been shown to increase the permeability of the BBB and blood-CSF barrier to various compounds [8, 21, 22], and could produce thereby indirect CNS effects. Other peptides, such as angiotensin, exert effects via receptors located in the circumventricular organs [6,13]. A similar mechanism involving the area postrema has been suggested for α-MSH in modulating central dopamine systems [11]. An understanding of the central actions of α-MSH therefore requires a critical evaluation of these various mechanisms.

There is no doubt that peripherally administered α-MSH penetrates into brain tissue and CSF [3, 7, 9, 19], though the degree to which this occurs is uncertain. One report has described low concentrations in CSF consistent with a low permeability at the blood-CSF barrier [3], while another suggested a significant permeability of the BBB into tissue [7]. The methodology employed in the latter report has, however, been questioned, and it may be too insensitive to quantify the uptake of peptides with only moderate BBB permeabilities [2,20]. Alternative techniques monitoring brain peptide concentrations over longer time periods have successfully measured permeabilities of other pro-opiocortin peptides [20]. The present experiments, conducted over similar extended time periods, have quantified the permeability of the blood-CSF barrier to α-MSH. Comparisons were made to the carbohydrate polymer inulin, a compound to which the blood-CSF barrier has only limited permeability.

METHOD

Male Wistar rats (380–520 g) were anesthetized by intraperitoneal injection of 0.24 mmol sodium pentobarbitone (Sagatal, May and Baker Ltd., Dagenham, England). They were prepared with carotid, jugular and tracheal cannulae for blood pressure monitoring, drug administration and blood sampling, and artificial ventilation respectively. Artificial CSF [16], preheated to 37°C, was injected at 63 μl/min via a 27 gauge stainless steel cannula into the right lateral ventricle [15], using a slow injection apparatus (C.F. Palmer, London, England). CSF perfusate overflowed and was collected from a second 21 gauge cannula implanted into the cisterna magna [1]. Animals were allowed to stabilize for approximately 15 min before the start of the experiment. After this time the

CSF perfusate was the artificial medium, endogenous CSF having been displaced.

Six rats were injected intravenously with a mixture of 30 nmol (50 μg) synthetic α-MSH (Cambridge Research Biochemicals Ltd., Harston, England) dissolved in 0.005 N HCl and 0.185 MBq inulin (^{14}C) carboxylic acid (specific activity 185 MBq/mmol, molecular weight 5200, Amersham International plc, Amersham, England) dissolved in distilled water. Six control animals received the inulin alone. Duplicate 10 μl and single 20 μl heparinized plasma samples were collected as described previously [28] before and up to 45 min after the injections for the determination of α-MSH and inulin concentrations respectively. A maximum of 13 blood samples was taken from any one animal. The CSF perfusate was collected in aliquots of 2 min duration into polystyrene tubes, immediately frozen, and stored at −20°C until assayed. Duplicate 20 μl samples of each aliquot of perfusate were used for measurement of α-MSH and the remainder, usually some 70 μl, or in certain cases the entire aliquot, for inulin determination

The α-MSH content of the plasma samples was measured by a specific radioimmunoassay in 1/4 to 1/32 or a higher series of dilutions as necessary [24–26]. That of CSF samples was measured similarly in a 1/2 to 1/16 series of dilutions.

Inulin was measured by scintillation counting in Pico-Fluor 30 (Packard Instrument Co., Downers Grove, Illinois, USA). Quench correction was performed using the external standard ratio method.

The plasma concentration data for α-MSH were fitted to a two-compartment open model where the concentration C in the plasma compartment at time t after the injection was described by the equation:

$$C_t = Ae^{-at} + Be^{-bt}$$

The four unknown parameters A, a, B and b were estimated by a non-linear least-squares minimization procedure with weighting equal to the reciprocal of the plasma concentration squared [12]. Plasma concentrations were corrected by subtraction of preinjection levels of endogenous α-MSH. Inulin plasma concentration data were fitted to a similar two-compartment closed model where the rate constant b was zero. The closed model was necessary as inulin, unlike α-MSH, is not metabolized and a clearance rate constant "b" could not be estimated from the data collected over the duration of the present experiments.

CSF concentration data for both α-MSH and inulin were fitted to the same one-compartment open model with an input from the plasma compartment at rate j and a clearance at rate k. The equation describing the CSF concentration C at time t after the injection for this model was:

$$C_t = \frac{Aje^{-at}}{k-a} + \frac{Bje^{-bt}}{k-b} - \left[\frac{Aj}{k-a} + \frac{Bj}{k-b}\right] e^{-kt}$$

This assumes that C_t at zero time was zero. CSF concentrations of α-MSH were therefore corrected by subtraction of the measured preinjection level. The unknown parameters A, a, B and b were set at the values determined from the plasma data for each animal, and the unknown parameters j and k estimated by the weighted least-squares minimization procedure described.

Differences between mean parameter estimates were detected by one-way analysis of variance followed by Duncan's multiple range test. Mean values are quoted in the paper as mean±S.E.M.

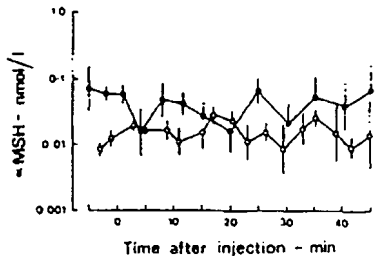

FIG. 1. Variations in the concentration of immunoreactive α-MSH in plasma (●) and CSF perfusate (○) after an intravenous injection of 0.185 MBq inulin (^{14}C) carboxylic acid. The points are the geometric means±S.E.M. resulting from pooling the data from five rats.

RESULTS

The variations in the concentration of endogenous α-MSH measured in the plasma and CSF perfusate of the control rats receiving inulin injections alone are shown in Fig. 1. Taken overall, the mean plasma concentration of 88.5±12.7 pmol/l was significantly higher ($p<0.001$, t-test) than the mean CSF concentration of 24.8±3.3 pmol/l. Fluctuations in plasma concentration observed during the experiments were in the normal range for anesthetized rats [25,28]. Concentrations of hormone in CSF were significantly less variable than in plasma ($p<0.001$, variance ratio test) but showed the tendency to increasing variability with time noted in other studies [16].

The increases in α-MSH concentration in plasma and CSF perfusate resulting from the intravenous peptide injection are shown in Fig. 2. The plasma data showed a biexponential decline in peptide concentration which was satisfactorily modelled by a two-compartment open system. The mean rate constant estimates obtained from separate fits to the data from each animal are given in Table 1. The half-lives for the two phases estimated from the mean rate constants were 2.3 and 14.2 min. The mean rate constant "b" was significantly different from the value of zero adopted for the plasma inulin data ($p<0.05$, t-test).

The α-MSH concentration data for the CSF perfusate showed a rapid but very limited rise in peptide levels (Fig. 2). The mean fitted peak concentration of 0.174±0.044 nmol/l occurred 1.68±0.29 min after the injection, at which time it was less than 0.05% of the plasma concentration. Thereafter, levels declined rapidly, and became indistinguishable from endogenous concentrations by on average 26 min after the injection. Computer modelling was therefore restricted to the time period when elevated concentrations were observed, and analysis of inulin data described below was restricted to the same range of data to maintain comparability. The single-compartment open model for the CNS provided a satisfactory fit to the α-MSH data. The mean rate constant estimates describing the input and output for this compartment, obtained from fits to the individual animal data, are given in Table 1. The rate constants are equivalent to an input with $t_{1/2}$=795 min and a clearance with $t_{1/2}$=0.5 min.

Inulin concentration data for both plasma and CSF perfusate were similar for both inulin-only and inulin + α-MSH injected groups of rats. The data for the inulin + α-MSH injected animals are displayed graphically in Fig. 2. Plasma concentrations declined in a biphasic manner which was described satisfactorily by the closed model. The mean esti-

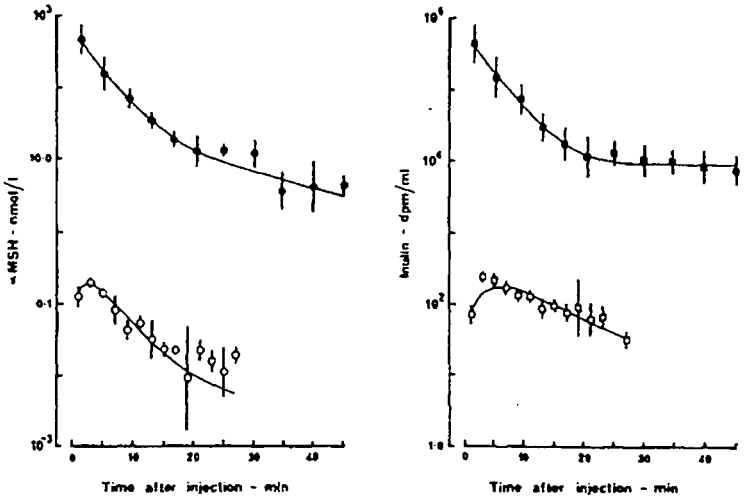

FIG. 2. Increases in the concentration of immunoreactive α-MSH in plasma (●) and CSF perfusate (○), and of radioactivity in plasma (■) and CSF perfusate (□) after simultaneous intravenous injection of 30 nmol synthetic α-MSH and 0.185 MBq inulin (^{14}C) carboxylic acid. The points are the geometric means±S.E.M. resulting from pooling the data from six rats. The curves are those obtained by fitting two-compartment models to the mean plasma data and one-compartment models to the mean CSF data.

TABLE 1

RATE CONSTANTS FOR THE DISTRIBUTION AND ELIMINATION OF α-MSH AND INULIN IN PERIPHERAL AND CNS COMPARTMENTS*

Drug	Injection combination	a† (min^{-1})	b† (min^{-1})	j‡ (min^{-1})	k§ (min^{-1})
Inulin	Inulin alone	0.174 ± 0.009	—	0.00126 ± 0.00075	0.118 ± 0.041
Inulin	Inulin + α-MSH	0.237 ± 0.026	—	0.00055 ± 0.00028	0.224 ± 0.094
α-MSH	Inulin + α-MSH	0.305 ± 0.030	0.049 ± 0.015	0.00087 ± 0.00034	1.30 ± 0.23

*Values expressed as mean ± S.E.M. (n=6).
†Hybrid constants for the two-compartment peripheral system.
‡Rate constant for transfer from the peripheral plasma compartment to the CNS compartment.
§Rate constant for elimination from the CNS compartment.

mates of rate constant "a" obtained from modelling the individual animal data are given in Table 1. The estimates for the inulin alone and inulin + α-MSH groups were not significantly different ($p>0.05$), and were equivalent to $t_{1/2}$ values of 4.0 and 2.9 min respectively. A comparison between the "a" constants for inulin and α-MSH when injected together was also non-significant ($p>0.05$).

Concentrations of inulin appearing in the CSF perfusate followed a pattern very similar to that observed for α-MSH (Fig. 2). Levels rose rapidly to reach a fitted peak concentration 5.5±0.7 min after the injection, when concentrations were less than 0.06% of the plasma concentration. Over the time period modelled, the inulin levels then declined, and this pattern could again be described adequately by the one-compartment open model. The mean rate constant estimates for both sets of inulin data, obtained from separate fits to the data for each rat, are given in Table 1. The inward rate constants were equivalent to processes with half-lives of 550 and 1257 min for the separate and combined injections, while the clearance constants gave $t_{1/2}$ values of 5.9 and 3.1 min respectively. Comparisons of the CNS compartment rate constants between inulin alone and inulin in combination with α-MSH were not significant for either the inward or outward processes ($p>0.1$). The critical comparison between the rate constant for movement from plasma into the CNS between α-MSH and inulin in the same animals was also non-significant ($p>0.1$). The rate of loss of α-MSH from the CNS compartment was, however, significantly greater than that of inulin ($p<0.01$).

DISCUSSION

The results demonstrate that following an intravenous bolus injection of α-MSH, immunoreactive peptide can be detected in CSF. The rise in α-MSH concentration in CSF was rapid but the occurrence of the peak concentration 1.68

min after the injection was a poor guide as to the rate of penetration which was calculated to have a half-life of over 13 hours. The early peak in CSF concentration was rather the result of the rapid distribution and elimination of the bolus dose in the periphery. As the plasma concentration driving the peptide into the CNS fell, the rapid CNS clearance ensured a corresponding fall in CSF concentration. The early peak in CSF inulin concentration will be the result of similar equilibration during the initial distribution of inulin in the periphery, CNS clearance for inulin being abnormally high owing to the perfusion system used. The blood-CSF barrier thus presents a considerable barrier to the penetration of α-MSH into CSF. The rate constant estimated for the movement from plasma into CSF was small, concentrations of injected peptide measured in the CSF perfusate always being less than 0.1% of plasma concentrations. The degree of this impermeability to α-MSH was assessed by comparison with inulin, a substance to which the blood-CSF barrier is known to have only limited permeability, and no significant difference between the rate of penetration of these two compounds into CSF could be demonstrated. The scale of the errors in the rate constant estimates were such that the rate of penetration of α-MSH would only have had to differ by 2.8 times from that of inulin to have reached significance ($p<0.05$). It seems probable, therefore, that α-MSH enters the CSF by ultrafiltration along with other water-soluble macromolecules with low permeabilities at cell membranes [13,18].

The latter conclusion is not affected by possible changes in the levels of endogenous α-MSH in plasma or CSF which were assumed to be constant during the experiment. Potential changes induced by α-MSH injection and not observed in the control animals would only serve to make a difference between α-MSH and inulin more likely. The α-MSH data do, however, represent immunological measurements and so the rate constants will refer to the behavior of a pool of intact and cross-reacting peptide fragments.

No influence of α-MSH on the permeability of the blood-CSF barrier was observed. The permeability to inulin was similar in both control and peptide injected groups. The dose, timing and the route of administration employed were not, however, favorable for demonstration of this phenomenon, which was reported for inulin after intracisternal injections of α-MSH [8, 21, 22].

The only significant difference in pharmacokinetic parameters observed was in the rate of clearance of α-MSH and inulin. The high rate of clearance of α-MSH from CSF indicates significant sequestration or metabolism of peptide by neural or glial components. The present data are therefore unable to shed any light on the permeability of the BBB to α-MSH. The lack of a difference in permeability observed between α-MSH and inulin rules out any significant contribution to CSF concentrations by α-MSH crossing the BBB, but cannot distinguish between low permeability at the BBB or higher permeability with subsequent sequestration/metabolism by brain tissue.

It is finally possible to estimate from the present data the contribution that blood-borne α-MSH makes to concentrations in CSF. Taking the mean plasma level in non-stressed rats to be 52.4 pmol/l [27], then the equilibrium CSF concentration can be calculated from the rate constant for entry into the CSF and an estimate of the clearance rate from CSF. The present clearance constant is unsuitable as it applies to a perfused system but, using the clearance rate constant of 0.0208 min^{-1} measured in an intact CSF system in the rat [3], the equilibrium level of α-MSH in CSF would be 2.2 pmol/l. This value is 9.3% of the mean CSF concentration of 23.7 pmol/l of endogenous α-MSH, which we have measured in other studies in natural CSF samples taken over a 24 hr period. The contribution of blood-borne α-MSH to CSF levels would thus appear to be small. The systemic circulation is not, however, the only route whereby pituitary α-MSH may reach the brain. The close anatomical relationship between the pituitary gland and the median eminence region may provide a route of entry into the brain independent of the systemic circulation [14]. The latter route could make contributions to CSF concentrations of α-MSH additional to those accounted for in the calculation above.

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ORIGINAL ARTICLE

LDL-C/HDL-C ratio in subjects with cardiovascular disease and a low HDL-C: results of the RADAR (Rosuvastatin and Atorvastatin in different Dosages And Reverse cholesterol transport) study

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Key words: Cardiovascular disease - High-density lipoprotein cholesterol - LDL-C/HDL-C ratio - Rosuvastatin

ABSTRACT

Background: The ratio of low-density lipoprotein cholesterol and high-density lipoprotein cholesterol (LDL-C/HDL-C) is a reliable predictor of cardiovascular risk. Low HDL-C levels in patients with coronary artery disease are associated with a high risk for cardiovascular events.

Objectives: This study compared the effects of rosuvastatin and atorvastatin on the LDL-C/HDL-C ratio in patients with cardiovascular disease and low HDL-C.

Methods: Patients aged 40–80 years with established cardiovascular disease and HDL-C < 1.0 mmol/L (< 40 mg/dL) entered a 6-week dietary run-in period, before randomisation to open-label treatment with rosuvastatin 10 mg (n = 230) or atorvastatin 20 mg (n = 231) for 6 weeks. Doses were increased after 6 weeks to rosuvastatin 20 mg or atorvastatin 40 mg, and after 12 weeks to rosuvastatin 40 mg or atorvastatin 80 mg. Serum lipid parameters were measured at baseline and 6, 12 and 18 weeks.

Results: After 6 weeks of treatment, mean percentage change from baseline in LDL-C/HDL-C ratio was –47.0% in the rosuvastatin group and –41.9%

in the atorvastatin group ($p < 0.05$ for between-group comparison). After 12 and 18 weeks of treatment, change from baseline was –53.0% and –57.3%, respectively, for rosuvastatin, compared with –47.9% and –49.6%, respectively, for atorvastatin ($p < 0.01$ and $p < 0.001$, respectively, for between-group comparison). Rosuvastatin also reduced LDL-C, total cholesterol and non-HDL-C significantly more than atorvastatin at all three time points, and significantly improved total cholesterol/HDL-C and apolipoprotein B/A-I ratios.

Conclusions: Rosuvastatin 10, 20 and 40 mg is significantly more effective than atorvastatin 20, 40 and 80 mg, respectively, in improving the LDL-C/HDL-C ratio in patients with cardiovascular disease and low HDL-C. Further studies are required to clarify the benefits of rosuvastatin for reduction of cardiovascular risk.

Introduction

Low-density lipoprotein cholesterol (LDL-C) is the primary target of lipid-modifying therapy for the prevention of cardiovascular disease, and international guidelines set out target levels for LDL-C reduction[1,2]. Statins are first-line therapy for reducing LDL-C levels, and clinical trials have demonstrated unequivocally that lowering LDL-C with statins significantly reduces the risk from cardiovascular disease in a wide range of patient types[3,4]. In addition to LDL-C, the contribution of other lipid fractions to the development of cardiovascular disease is now increasingly recognised. Epidemiological studies have repeatedly shown an inverse relationship between serum high-density lipoprotein cholesterol (HDL-C) levels and the risk from coronary artery disease (CAD)[5-7]. Although no specific goal for HDL-C has been defined, current European guidelines consider HDL-C < 1.0 mmol/L (40 mg/dL) in men and < 1.2 mmol/L (46 mg/dL) in women to be a risk factor for cardiovascular disease[2]. In addition, a UK consensus group has recommended a HDL-C level of ≥ 1.0 mmol/L for all patients with cardiovascular disease or diabetes[8].

Several types of lipid-modifying agents are able to increase levels of HDL-C, including fibrates and statins. The effects of raising HDL-C with statins on cardiovascular risk reduction have not been specifically investigated, although post-hoc analyses of the Scandinavian Simvastatin Survival Study database have reported that a 10% increase in HDL-C was associated with a statistically significant 8% decrease in the risk from major coronary events[9], and that the beneficial effects of simvastatin were more pronounced in patients with low HDL-C and elevated triglycerides at baseline[10].

Given that the beneficial effects of raising HDL-C and lowering LDL-C may be additive, use of an agent with optimal effects on both these parameters may increase cardiovascular risk reduction. Statins may decrease levels of LDL-C as well as raising levels of HDL-C, although the magnitude of these effects is both drug- and dose-dependent[11]. Recent studies have demonstrated that rosuvastatin, followed by atorvastatin, is the most effective statin for reducing LDL-C[12,13]. In addition, while the efficacy for raising HDL-C is maintained across the dose range for rosuvastatin, HDL-C response has been shown to diminish with increasing doses of atorvastatin[12,14]. Statins also have variable effects on the ratio of LDL-C/HDL-C[15]. This ratio is a powerful predictor of cardiovascular risk and may provide additional predictive power compared with LDL-C alone[16,17]. Low HDL-C levels in patients with CAD are associated with a high risk for cardiovascular events[18]. Therefore, the present study compared the effects of rosuvastatin and atorvastatin on the LDL-C/HDL-C ratio in patients with established cardiovascular disease and low HDL-C levels.

Methods

Study design

The RADAR (Rosuvastatin and Atorvastatin in different Dosages And Reverse cholesterol transport) study was an open-label, randomised, parallel-group, multicentre, phase IIIb study conducted in 29 centres in The Netherlands. The study was designed and conducted in accordance with the Declaration of Helsinki and in compliance with the ethical principles of Good Clinical Practice. Appropriate ethics committees or institutional review boards approved the study, and all patients gave written, informed consent.

Enrolled patients entered a 6-week dietary run-in phase during which they were instructed to follow the National Cholesterol Education Program step 1 diet. At the end of the run-in period (week 0; baseline), eligible patients continued to follow the diet and were randomised sequentially (in blocks of 4) to receive rosuvastatin 10 mg or atorvastatin 20 mg for 6 weeks. At week 6, the doses were increased to rosuvastatin 20 mg or atorvastatin 40 mg for a further 6 weeks, and at week 12 the doses were increased to rosuvastatin 40 mg or atorvastatin 80 mg for a further 6 weeks, making 18 weeks' treatment in total (Figure 1).

The primary endpoint of the study was the percentage change from baseline to 6 weeks in the LDL-C/HDL-C ratio. Secondary endpoints were the percentage change in the LDL-C/HDL-C ratio from baseline to 12 and 18 weeks and the percentage change from baseline in apolipoprotein B/A-I ratio after 6, 12 and 18 weeks

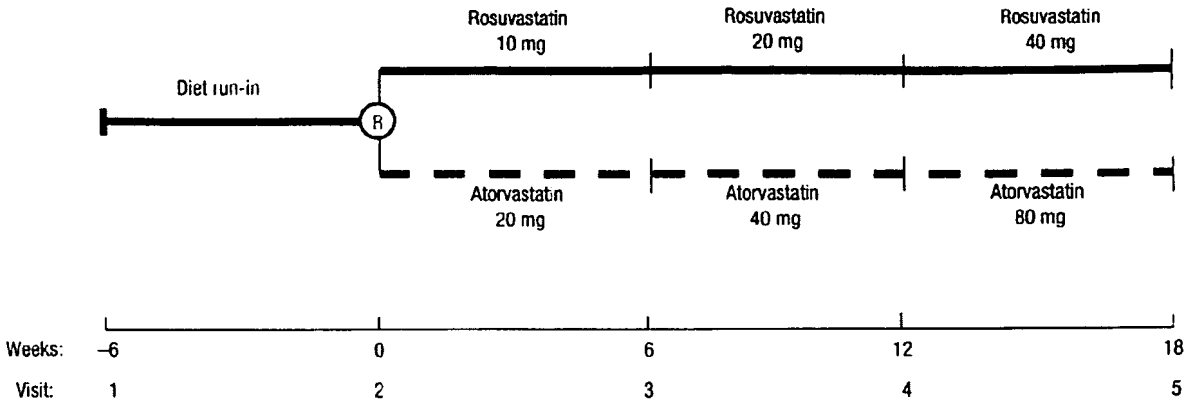


Figure 1. RADAR study design

of treatment. Tertiary endpoints included: percentage change from baseline to 6, 12 and 18 weeks in HDL-C, LDL-C, non-HDL-C, total cholesterol/HDL-C ratio, apolipoprotein B, apolipoprotein A-I, triglycerides and total cholesterol. Post-hoc analyses were also performed to determine the proportion of patients achieving LDL-C goals of < 2.5 mmol/L (100 mg/dL) and < 2.0 mmol/L (80 mg/dL), as advocated recently by the third joint European task force and British Hypertension Society, respectively[2,19].

Study population

Men or women aged 40–80 years were eligible for participation in the study if they had established cardiovascular disease, fasting HDL-C concentrations of < 1.0 mmol/L (40 mg/dL) at visit 1 and baseline, and triglycerides ≤ 4.5 mmol/L (400 mg/dL) at visit 1. Established cardiovascular disease was defined as one or more of the following: a history of transient ischaemic attack or ischaemic stroke; carotid artery disease; advanced atherosclerosis (≥ 60% stenosis) in the common or internal carotid artery; CAD (defined by a history of myocardial infarction or hospitalisation for unstable angina, angina pectoris with objective evidence of myocardial ischaemia, coronary revascularisation or angiographic evidence of > 50% stenosis in one or more major epicardial coronary artery); or peripheral arterial disease (defined by a history of aortic aneurysm repair, arterial surgery or angioplasty performed to relieve lower limb ischaemia, lower limb amputation performed due to complications of atherosclerotic arterial disease, or intermittent claudication with an ankle–brachial pressure index < 0.9).

Patients were excluded from the study if they met any of the following criteria: use of lipid-lowering drugs (including nicotinic acid), dietary supplements or food additives after enrolment; history of hypersensitivity to statins; pregnancy, lactation or childbearing potential without reliable contraceptive use; active arterial disease (unstable angina, myocardial infarction, transient ischaemic attack, cerebrovascular accident, coronary artery bypass graft or angioplasty) within 2 months of entry into the dietary lead-in phase; likely requirement for therapeutic coronary artery intervention within 6 months of randomisation; uncontrolled hypertension; glycated haemoglobin > 8% at enrolment; history of malignancy; uncontrolled hypothyroidism; homozygous familial hypercholesterolaemia or type III hyperlipoproteinaemia; history of alcohol and/or drug abuse; active liver disease; serum creatinine > 180 μmol/L at enrolment; unexplained creatine kinase > 3 times the upper limit of normal at enrolment; received an investigational drug within 4 weeks before enrolment; serious or unstable medical or psychological conditions that could, in the opinion of the investigator, compromise the subject's safety or successful participation in the trial. Concomitant treatment with erythromycin, clarithromycin, azole antifungal agents, cyclosporin, antiviral agents (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir), phenytoin, carbamazepine, phenobarbital, nefazodone, or lipid-lowering therapy (other than study medication) was prohibited during the trial.

Measurements and statistical analyses

Blood samples were collected at the start of the run-in period, at randomisation (baseline), and after 6, 12 and 18 weeks of treatment. Patients were required to fast for at least 12 h before blood collection. Lipid and lipoprotein analyses were conducted at the local hospital laboratory using the COVANCE Virtual Central Laboratory concept, except for apolipoprotein B and apolipoprotein A-I, which were measured at a central laboratory. Levels of LDL-C were calculated using the Friedewald equation for patients with triglycerides ≤ 4.5 mmol/L (400 mg/dL), and measured by β-quantification for those with

triglycerides > 4.5 mmol/L. Levels of non-HDL-C were calculated by subtracting HDL-C from total cholesterol.

The primary endpoint was compared between groups using analysis of variance, with a factor fitted for treatment. The effects of treatment are illustrated by calculating mean percentage change from mean baseline levels and presented as least squares means. Secondary and tertiary endpoints were analysed in the same way. The post-hoc analyses of the proportion of patients achieving LDL-C goals were carried out using logistic regression with factors fitted for baseline LDL-C values and treatment. All efficacy analyses were performed using data from the intention-to-treat population, consisting of all patients with baseline data, using the last observation carried forward approach. In addition, a sensitivity analysis was conducted for the primary endpoint, excluding patients from the intention-to-treat population with missing data on the LDL-C/HDL-C ratio at 6 weeks. The safety analysis set included all subjects who received at least one dose of study medication.

A 5% difference between treatments in percentage change from baseline for the primary endpoint, LDL-C/HDL-C ratio, was predefined as clinically meaningful. Assuming a standard deviation of 17%, and to show the 5% difference with a power of 90% and a two-sided significance level of 5%, it was estimated that approximately 250 patients needed to be randomised to each treatment group to provide data on 238 patients.

Safety assessments included recording of any adverse event using a standard question at each study visit after enrolment. An adverse event was classified as serious if it met one or more of the following criteria: fatal; life-threatening; required inpatient hospitalisation or prolongation of existing hospitalisation; resulted in persistent or significant disability or incapacity; congenital abnormality or birth defect; or an important medical event that could jeopardise the patient or could require medical intervention to prevent one of the above outcomes. Laboratory safety measurements included aspartate aminotransferase, alanine aminotransferase, creatinine, and creatine kinase, and were performed by the local hospital laboratory using the COVANCE Virtual Central Laboratory concept. All randomised patients who received at least one dose of study medication were evaluated for safety, and data are summarised descriptively without statistical analysis.

Results

Patient characteristics

A total of 461 patients were randomised, 230 to rosuvastatin and 231 to atorvastatin. Baseline patient characteristics were very similar between the two treatment groups (Table 1). The use of concomitant medications in the rosuvastatin and atorvastatin groups was similar throughout the study period (angiotensin-

Table 1. Patient demographics and baseline characteristics

	Rosuvastatin (n = 230)	Atorvastatin (n = 231)
Gender, male/female (%)	209/21 (91/9)	209/22 (90/10)
Race, white (%)	230 (100)	229 (99)
Mean (SD) age (years)	60.7 (9.3)	60.2 (9.4)
Mean (SD) weight (kg)	88.6 (14.2)	88.5 (13.4)
Mean (SD) body mass index (kg/m^2)	28.7 (4.08)	28.9 (3.91)
Mean (SD) systolic blood pressure (mmHg)	139 (21.6)	137 (19.4)
Mean (SD) diastolic blood pressure (mmHg)	80.8 (8.8)	80.1 (8.8)
Lipids		
Mean (SD) LDL-C/HDL-C	4.8 (1.5)	4.8 (1.7)
Mean (SD) LDL-C (mmol/L)	3.6 (1.2)	3.7 (1.3)
Mean (SD) TC (mmol/L)	5.8 (1.3)	5.7 (1.3)
Mean (SD) HDL-C (mmol/L)	0.8 (0.1)	0.8 (0.1)
Mean (SD) triglycerides (mmol/L)	2.8 (1.5)	2.7 (1.4)
Prior cardiovascular disease		
Transient ischaemic attack (%)	5	5
Ischaemic stroke (%)	5	3
Carotid artery disease (%)	3	2
Angina pectoris (%)	83	80
Myocardial infarction (%)	62	65
Peripheral arterial disease (%)	4	4

To convert cholesterol in mmol/L to mg/dL, multiply by 38.7; to convert triglycerides in mmol/L to mg/dL, multiply by 88.6

SD = standard deviation; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; TC = total cholesterol

converting enzyme inhibitors 31% vs 35%, beta-blocking agents 64% vs 64%, dihydropyridine derivatives 21% vs 23%, platelet aggregation inhibitors 86% vs 87%, vitamin K antagonists 13% vs 11% and thiazides 2% vs 1% in the rosuvastatin and atorvastatin groups, respectively). A total of eight patients in the rosuvastatin group and 10 patients in the atorvastatin group discontinued from the study. The intention-to-treat population analysed for efficacy contained 230 patients in the rosuvastatin group and 231 in the atorvastatin group. The safety population comprised 229 and 230 patients in the rosuvastatin and atorvastatin groups, respectively (one randomised patient in each group failed to receive study medication and was therefore excluded from the safety population).

Efficacy

Rosuvastatin 10 mg improved the LDL-C/HDL-C ratio at 6 weeks significantly more than atorvastatin 20 mg (mean [SE] change from baseline −47.0% [1.6] vs −41.9% [1.6], $p < 0.05$) (Figure 2). Results from the sensitivity analysis, excluding patients from the intention-to-treat population with missing data on the LDL-C/HDL-C ratio at 6 weeks, confirmed the findings of the primary endpoint ($p < 0.05$). Significantly greater improvements in the LDL-C/HDL-C ratio were also observed for rosuvastatin 20 mg compared with atorvastatin 40 mg at 12 weeks (−53.0% [1.4] vs −47.9% [1.4], $p < 0.01$), and for rosuvastatin 40 mg compared with atorvastatin 80 mg at 18 weeks (−57.3% [1.4] vs −49.6% [1.4], $p < 0.001$).

Table 2 summarises the changes from baseline in other lipid and lipoprotein parameters at 6, 12 and 18 weeks. Rosuvastatin reduced LDL-C, the total cholesterol/HDL-C ratio, total cholesterol, non-HDL-C, and the apolipoprotein B/A-I ratio significantly more than atorvastatin at all three time points. Similar increases in HDL-C were observed with rosuvastatin 10 mg and atorvastatin 20 mg at 6 weeks; at 12 and 18 weeks, increases in HDL-C were larger with rosuvastatin than

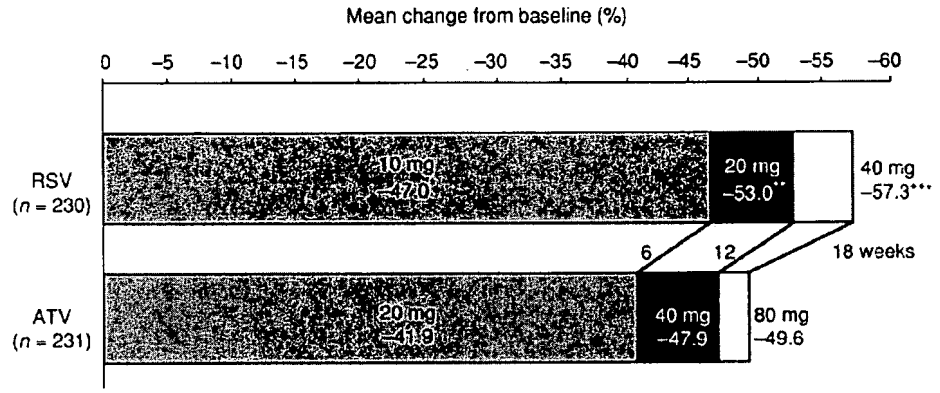


Figure 2. Mean percentage change from baseline in LDL-C/HDL-C ratio after 6, 12 and 18 weeks' treatment with rosuvastatin and atorvastatin. LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; RSV = rosuvastatin; ATV = atorvastatin

Table 2. Mean percentage change from baseline (SE) in lipid and lipoprotein parameters at 6, 12 and 18 weeks

Lipid/lipoprotein	6 weeks		12 weeks		18 weeks	
	RSV 10 mg	ATV 20 mg	RSV 20 mg	ATV 40 mg	RSV 40 mg	ATV 80 mg
LDL-C	−44.0 (1.5)*	−38.4 (1.5)	−50.4 (1.3)**	−45.1 (1.3)	−55.3 (1.2)****	−48.1 (1.2)
HDL-C	+3.9 (1.0)	+4.1 (1.0)	+5.5 (1.0)	+3.1 (1.0)	+4.7 (1.1)	+2.7 (1.1)
TC	−37.4 (0.7)****	−32.5 (0.7)	−41.1 (0.8)***	−37.3 (0.8)	−44.7 (0.9)****	−39.5 (0.9)
Triglycerides	−29.2 (1.8)*	−23.9 (1.8)	−32.2 (1.8)	−27.3 (1.8)	−35.4 (1.8)	−31.6 (1.8)
TC/HDL-C	−40.3 (0.8)****	−35.6 (0.8)	−44.6 (0.9)****	−39.5 (0.9)	−47.8 (1.0)****	−41.5 (1.0)
Apolipoprotein B	−28.0 (2.4)	−24.0 (2.5)	−30.6 (2.4)	−24.5 (2.4)	−34.3 (2.3)	−28.3 (2.3)
Apolipoprotein A-I	+4.6 (1.6)	+2.7 (1.7)	+7.7 (2.0)	+5.7 (2.0)	+8.6 (2.3)	+3.3 (2.3)
Apolipoprotein B/A I	35.5 (1.0)**	−30.9 (1.0)	−40.0 (1.1)**	−35.1 (1.1)	−43.3 (1.1)****	−36.3 (1.2)
Non-HDL-C	−44.3 (0.8)****	−38.8 (0.8)	−49.0 (0.8)****	−44.2 (0.8)	−53.0 (1.0)****	−46.7 (1.0)

SE = standard error; RSV = rosuvastatin; ATV = atorvastatin; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; TC = total cholesterol
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (all comparisons vs atorvastatin at the same time point)

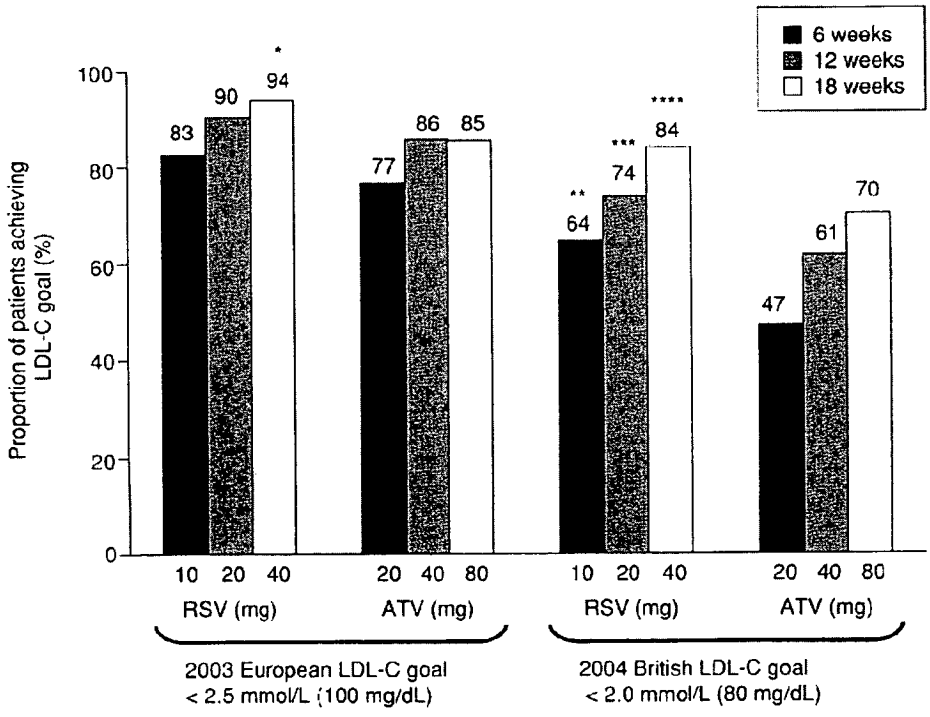
with atorvastatin, but differences between treatments did not reach statistical significance. Greater reductions in triglycerides were observed with rosuvastatin compared with atorvastatin at 6, 12 and 18 weeks, reaching statistical significance for the rosuvastatin 10 mg vs atorvastatin 20 mg comparison at 6 weeks. Both treatments increased apolipoprotein A-I and decreased apolipoprotein B levels, but the between-group differences were not statistically significant for either parameter at any time point.

Overall, a greater proportion of patients treated with rosuvastatin achieved the 2003 European LDL-C goal of < 2.5 mmol/L (100 mg/dL) compared with atorvastatin (Figure 3); the difference between treatment groups reached statistical significance at week 18 (94% vs 85%, $p < 0.01$). Similarly, a greater proportion of patients receiving rosuvastatin achieved the more stringent LDL-C goal of < 2.0 mmol/L (80 mg/dL) (Figure 3), with statistically significant differences between treatment groups in favour of rosuvastatin at 6 weeks (64% vs 47%, $p < 0.0001$), 12 weeks (74% vs 61%, $p < 0.01$) and 18 weeks (84% vs 70%, $p < 0.001$).

An additional post-hoc analysis was performed to assess differences in treatment effects across the whole study period for HDL-C, apolipoprotein B, apolipoprotein A-I and the apolipoprotein B/A-I ratio (Figure 4). The analysis used a mixed-effects model with factors fitted for treatment, time and treatment-time interaction. No significant difference between treatments over time was identified for HDL-C and apolipoprotein B. However, the differences were significant for effects on apolipoprotein A-I ($p = 0.004$) and the apolipoprotein B/A-I ratio ($p = 0.002$).

Tolerability

Both treatments were well tolerated up to maximal recommended doses in this forced-titration study. The overall occurrence of adverse events associated with each treatment was similar, and the occurrence of deaths, serious adverse events and withdrawals due to adverse events was low, with no differences noted between treatment groups. Two patients died during the study, one from sudden death (rosuvastatin group) and one from liver metastasis (atorvastatin group), with neither death considered related to study treatment. Two treatment-related serious adverse events (both high creatine kinase activities) were reported, both in the atorvastatin group.



*$p < 0.01$ vs ATV 80 mg for LDL-C < 2.5 mmol/L; **$p < 0.0001$ vs ATV 20 mg for LDL-C < 2.0 mmol/L; ***$p < 0.01$ vs ATV 40 mg for LDL-C < 2.0 mmol/L; ****$p < 0.001$ vs ATV 80 mg for LDL-C < 2.0 mmol/L

Figure 3. Proportion of patients achieving LDL-C targets after 6, 12 and 18 weeks' treatment with rosuvastatin and atorvastatin. LDL-C = low-density lipoprotein cholesterol; RSV = rosuvastatin; ATV = atorvastatin

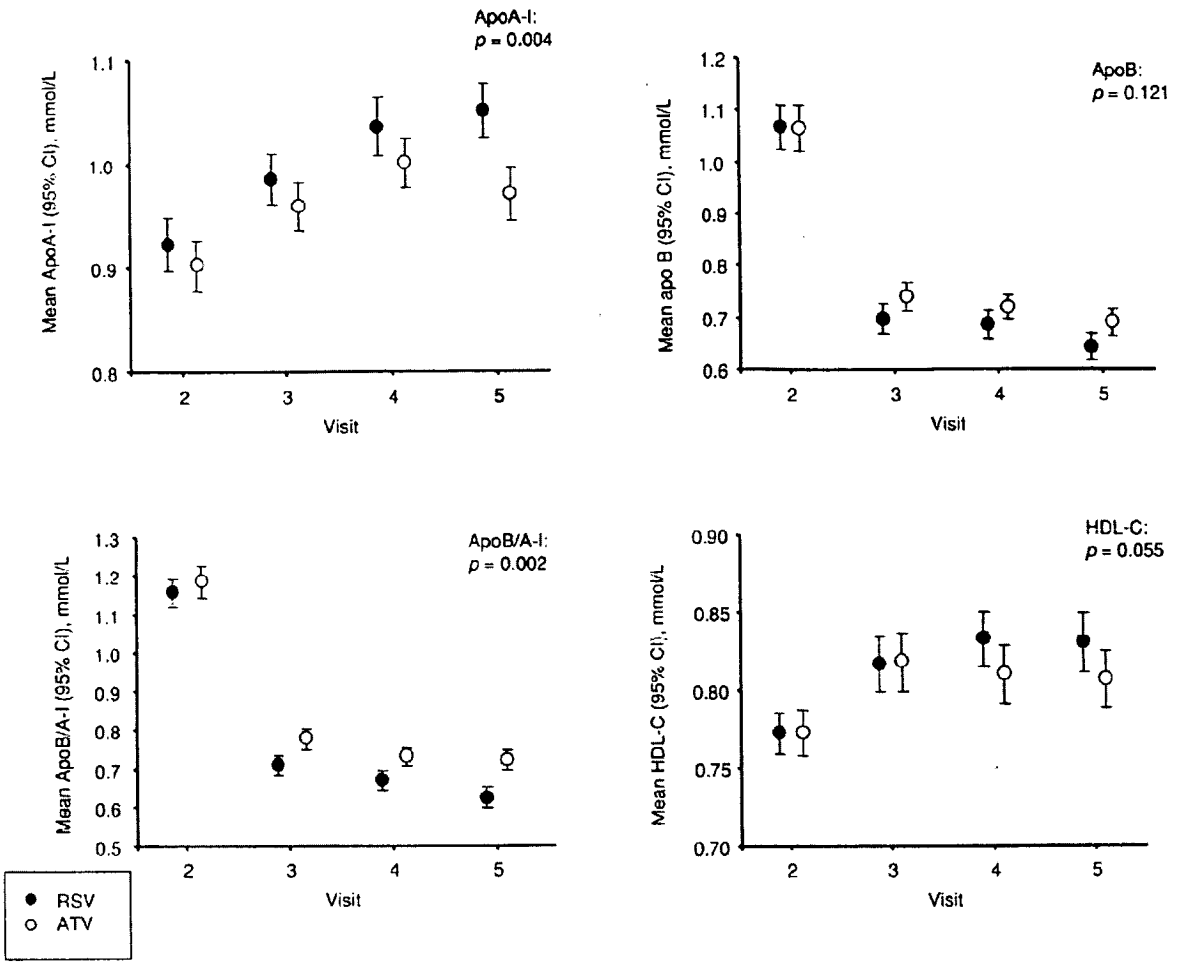


Figure 4. Treatment effects at 6, 12 and 18 weeks on HDL-C, ApoB, ApoA-I and the ApoB/A-I ratio with rosuvastatin and atorvastatin. HDL-C = high-density lipoprotein cholesterol; Apo = apolipoprotein; CI = confidence intervals; RSV = rosuvastatin; ATV = atorvastatin

The incidence of myalgia was similar in the two treatment groups (rosuvastatin group 7%, atorvastatin group 8%). No cases of myopathy (creatine kinase > 10 × upper limit of normal and muscle symptoms) were reported in either treatment group. One patient receiving atorvastatin had a clinically important increase in creatine kinase (> 10 × upper limit of normal); however, this was not accompanied by muscle symptoms. During the course of the study, serum creatinine levels decreased in both treatment groups, with no difference between treatments. In the rosuvastatin group, mean (SD) serum creatinine concentration was 101.7 (19.2) µmol/L at baseline; at the end of the study, mean (SE) percentage change from baseline was 8.5 (1.1) ($p < 0.0001$ vs baseline). In the atorvastatin group, mean (SD) serum creatinine concentration was 103.6 (20.3) µmol/L at baseline; at the end of the study, mean (SE) percentage change from baseline was 8.7 (1.0) ($p < 0.0001$ vs baseline). Glomerular filtration rate (GFR) was determined at baseline and the end of the treatment period using the Modified Diet in Renal Disease (MDRD) equation. A slight increase was noted in both treatment groups, with no difference between treatments. Mean (SE) percentage change from baseline after 18 weeks of treatment was 9.6 (1.2) in the rosuvastatin group ($p < 0.0001$ vs baseline) and 9.9 (1.2) in the atorvastatin group ($p < 0.0001$ vs baseline). There were no clinically important abnormalities in alanine aminotransferase or aspartate aminotransferase activity (> 3 × upper limit of normal) during the study.

Discussion

In the present study, rosuvastatin was more effective than atorvastatin in improving the LDL-C/HDL-C ratio in patients with established cardiovascular disease and low levels of HDL-C after 6, 12 or 18 weeks of treatment,

and this greater effect was achieved with lower doses. In this group of patients with cardiovascular disease and low HDL-C levels, treatment with rosuvastatin 10 mg appears to improve the LDL-C/HDL-C ratio to a similar extent as forced titration to atorvastatin 40 mg. Previous studies in patients with CAD, type 2 diabetes or hypercholesterolaemia have also demonstrated that rosuvastatin reduces the LDL-C/HDL-C ratio more effectively than equivalent and higher doses of other statins, including atorvastatin[15,20,21].

Rosuvastatin was also more effective than atorvastatin in reducing LDL-C, total cholesterol and non-HDL-C, and improving the total cholesterol/HDL-C and apolipoprotein B/A-I ratios, at all three time points. These results are consistent with those from previous studies that have compared the effects of rosuvastatin and atorvastatin in patients with hypercholesterolaemia[22]. Although rosuvastatin increased HDL-C numerically more than atorvastatin at 12 and 18 weeks of treatment, this difference did not reach statistical significance between the groups. Consistent with previous observations[12,14], the HDL-C response to atorvastatin in the present study diminished with increasing doses, albeit non-significantly. The greater reductions in LDL-C with rosuvastatin resulted in a greater proportion of patients achieving their LDL-C goals; even at the usual starting dose of 10 mg, more than 80% of patients treated with rosuvastatin achieved the 2003 European LDL-C goal of < 2.5 mmol/L (100 mg/dL).

While LDL-C remains the primary target for prevention of CAD, other lipid abnormalities such as low levels of HDL-C also contribute to CAD risk. Consequently, lipid measures such as the LDL-C/HDL-C ratio, which reflect the levels of multiple components, may improve risk prediction. For example, analysis of data from over 2000 patients in the placebo group of the Helsinki Heart Study showed that the LDL-C/HDL-C ratio was the best single predictor of cardiac events[23]. More recently, analysis of 5-year data from the Program on the Surgical Control of Hyperlipidaemias study found that the highest hazard ratios were for the LDL-C/HDL-C ratio[16]. The apparent prognostic benefit provided by this ratio may reflect the opposing effects of LDL-C and HDL-C on cholesterol transport. Particles of LDL-C carry cholesterol from the liver to the periphery, whereas HDL-C is involved in the reverse cholesterol transport mechanisms that remove cholesterol from artery walls and transport it back to the liver[24]. Thus, the ratio may reflect the balance between atherogenic and atheroprotective processes. Similarly, the ratios total cholesterol/HDL-C and apolipoprotein B/A-I also improve cardiovascular risk prediction[17]. For example, a study of 848 patients with angiographically proven CAD and successfully treated with statins (as defined by ≥ 30% decrease from baseline in total cholesterol) demonstrated no significant association between LDL-C and subsequent cardiovascular events[25]. However, the concentration of apolipoprotein B was predictive of subsequent events (hazard ratio 3.21, $p = 0.033$), and the ratio of apolipoprotein B/A-I was an even stronger predictor (hazard ratio 7.22, $p < 0.0001$). Similarly, in the AMORIS (Apolipoprotein-related MORtality RISk) study of more than 175 000 individuals followed up for 5.5 years, apolipoprotein B level was a highly significant predictor of fatal acute myocardial infarction, while the strongest univariate predictor was the ratio of apolipoprotein B/A-I[26]. These findings were valid for men and women, and, unlike more conventional lipid risk factors, in people 70 years and older. Recently, the worldwide INTERHEART study demonstrated that the apolipoprotein B/A-I ratio was the strongest predictor of myocardial infarction among nine risk factors, including smoking, hypertension and the presence of diabetes[27]. It is interesting to note that the present study showed a significantly greater improvement in the apolipoprotein B/A-I and total cholesterol/HDL-C ratios with rosuvastatin at all three time points.

Overall, the statin treatments were well tolerated in this study, with no obvious differences between treatment groups with regard to adverse events. Transient increases in hepatic transaminases and asymptomatic increases in creatine kinase were infrequent and not indicative of hepatotoxicity or myotoxicity. Treatment with rosuvastatin or atorvastatin appeared to improve renal function, consistent with previous longer-term studies with these agents[28,29].

Limitations of the current study include the open-label design, the lack of hard clinical endpoints and the relatively short duration. Despite the open-label design, the number and nature of adverse events were generally consistent with those observed in previous double-blind studies of rosuvastatin and atorvastatin[22]. In addition, although the study was designed to determine effects of treatment on lipid levels and not cardiovascular events, the lipid levels measured here serve as a marker of cardiovascular risk[16,23]. Also, since statins are known to have a rapid onset of action, the time points chosen for efficacy and safety measurements (6, 12 and 18 weeks) were sufficient to observe the maximal lipid effects for the studied drugs and doses.

Conclusions

In summary, rosuvastatin 10, 20 and 40 mg was significantly more effective than atorvastatin 20, 40 and

80 mg, respectively, in improving the LDL-C/HDL-C ratio in patients with cardiovascular disease and low HDL-C, in addition to significantly improving other components of the lipid profile. Further studies are required to clarify whether the beneficial effects of rosuvastatin on the lipid profile translate into cardiovascular risk reduction. However, use of a statin with greater benefits across the lipid profile may improve the management of dyslipidaemia in subjects with, or at risk of, cardiovascular disease, and should enable a greater proportion to achieve their lipid goals.

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The ratio of apoB/apoAI, apoB and lipoprotein(a) are the best predictors of stable coronary artery disease

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Abstract

Background: The ratio of low- to high-density lipoprotein-cholesterol (LDL-C/HDL-C) conventionally represents the balance of proatherogenic and antiatherogenic lipids. However, growing evidence supports the idea that the ratio of apolipoprotein (apo) B/apoAI is a better index for risk assessment of coronary artery disease (CAD). The aim of this study was to evaluate the efficiency of advanced profile of serum (apo)lipoproteins for predicting stable CAD in secondary prevention.

Methods: The study subjects, 138 men and 126 women aged 40–70 years, were classified as CAD cases or controls, according to the results of coronary angiography. The severity of CAD was scored on the basis of the number and extent of lesions in coronary arteries. Serum (apo)lipoproteins were measured by immunoturbidometric and electrophoresis methods.

Results: Patients with CAD compared with controls had increased serum levels of triglycerides (2.6 ± 2.0 vs. 2.0 ± 1.2 mmol/L, $p\leq0.005$), apoB (1.36 ± 0.31 vs. 1.19 ± 0.24 g/L, $p\leq0.0001$), lipoprotein(a) [Lp(a)] (0.69 ± 0.60 vs. 0.43 ± 0.31 g/L, $p\leq0.0001$) and apoB/apoAI ratio (1.07 ± 0.32 vs. 0.87 ± 0.18, $p\leq0.0001$), and decreased serum levels of HDL-C (1.02 ± 0.29 vs. 1.11 ± 0.34 mmol/L, $p\leq0.03$), apoAI (1.32 ± 0.22 vs. 1.37 ± 0.19 g/L, $p\leq0.04$) and LDL-C/apoB ratio (0.91 ± 0.32 vs. 1.02 ± 0.25 mmol/g, $p\leq0.01$). Multiple logistic regression analysis after adjusting for major risk factors showed that the apoB/apoAI ratio, apoB and Lp(a) were among seven significant and independent determinants of CAD. The area under the receiver operating characteristic (ROC) curves (AUC) as a relative measure of test efficiency was highest and significant for the apoB/apoAI ratio (AUC=0.71, $p\leq0.0001$), apoB (0.67, $p\leq0.0001$), Lp(a) (0.63, $p\leq0.001$), the LDL-C/apoB ratio (0.62, $p\leq0.006$), triglycerides (0.62, $p\leq0.004$) and apoAI (0.58, $p\leq0.05$). ANOVA analysis showed significant association for the apoB/apoAI ratio, apoB, Lp(a) and triglycerides, and moderate association for total cholesterol and its subfractions, with the severity of CAD.

Conclusions: The results indicate that the apoB/apoAI ratio, apoB and Lp(a) are independent risk factors for CAD and are superior to any of the cholesterol ratios. We suggest using the apoB/apoAI ratio as the best marker of CAD in clinical practice.
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Keywords: apolipoprotein (apo); apoB/apoAI ratio; cholesterol; coronary artery disease; lipoprotein(a).

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Introduction

Coronary artery disease (CAD) is a multifactorial disorder with over 250 different known risk factors (1). Advancing age, male sex, hypertension, diabetes mellitus, cigarette smoking and dyslipidemia are the major and independent risk factors for CAD (2). The classical profile of lipid abnormalities includes elevated serum total cholesterol and low-density lipoprotein cholesterol (LDL-C), and reduced high-density lipoprotein cholesterol (HDL-C). Although elevated plasma lipids should clearly be considered as an important risk indicator of cardiovascular disease (CVD), approximately half of patients with CAD do not exhibit any of the classical risk factors, and the excess risk for CAD remains unexplained (3). Therefore, additional (apo)lipoprotein measurements have been proposed to better identify patients potentially at risk for CAD. Measurement of apolipoprotein AI (apoAI), the major protein of HDL, has shown to offer advantages over HDL-C for assessment of CAD risk (4). ApoB, the main structural protein of atherogenic lipoprotein particles, is a strong predictor of CAD (5–7). Several large and multi-ethnic studies have reported that the apoB/apoAI ratio is superior to any of the conventional cholesterol ratios for atherosclerosis, myocardial infarction and stroke (8–11). Lipoprotein(a) [Lp(a)] has thrombotic properties and atherogenic capacity, and high plasma levels correlate with CVD (12–15). Nevertheless, the role of Lp(a) as a risk factor for atherosclerosis is still controversial (16–20).

Data for advanced profiles of serum lipids and (apo)lipoproteins in CAD patients of Iranian descent are too limited. Since these patients often use drugs that affect serum lipids, it is interesting to evaluate tests for better identification of stable CAD in secondary prevention. The current study was undertaken to investigate the efficiency and association of the advanced profile of serum lipids and (apo)lipoproteins with the occurrence and severity of angiographic defined CAD.

Materials and methods

The experimental design, angiographic assessment and anthropometric measurements were as described previously

(20). In brief, the study population consisted of 138 men and 126 women aged 40–70 years who were suspected of having CAD and underwent a first coronary angiography at Zahra Hospital, University of Mazandaran. Individuals with a recent history of acute myocardial infarction, precutaneous transluminal coronary angioplasty, or renal, infectious or malignant disease were excluded. Subjects with one or more lesions that narrowed the lumen of any coronary artery significantly (≥ 70%) were considered to be CAD cases, whereas those without any narrowing (< 10%) were taken as controls (7). The severity of coronary occlusion was scored on the basis of the number and extent of lesions as: normal, mild, moderate and severe (20–23).

Biochemical measurements

All measurements were carried out on fresh serum, except for apolipoproteins and Lp(a), for which samples were stored at −70°C for a maximum of 3 months before analysis. Serum lipids were measured as described previously (20). ApoB100, apoAI and Lp(a) were assayed by immunoturbidometric methods (DiaSys Diagnosis Inc., Holzheim, Germany) performed by Pars-Azmon. Assay performance was monitored every 20 tests using the lipid control sera, Tru-Lab Lipids and Tru-Lab Lp(a) (Diagnosis Inc.). Lipid standards Tru Cal apoB, apoAI and Lp(a) were used for daily calibration of a Cobas-Mira Plus auto-analyzer (Roche Diagnostic Systems, Basel, Switzerland). Inter- and intra-assay coefficients of variance (CVs) were 2.3% and 1.7% at 0.885 g/L apoAI, 2.5% and 1.8% for 0.952 g/L apoB, and 2.3% and 1.5% for 0.522 g/L Lp(a), respectively. Serum lipoproteins were fractionated by electrophoresis on agarose gel (Sebia, Issy-les-Moulineaux, France). Each serum sample was applied to the gel in duplicate and gels were stained using amido black after electrophoresis. The gels were scanned three times at 580 nm around the middle of electrophoretic bands and the mean was calculated. Using this procedure, intra- and inter-assay CVs for all lipoprotein fractions were less than 3% and 5.5%, respectively.

Statistical analysis

Results are presented as mean ± SD. Significant differences for categorical variables were assessed by χ^2 test and continuous variables by Student's t-test and analysis of variance (ANOVA). All p-values are two-tailed and differences were

Table 1 Demographic and biochemical parameters for CAD control subjects and CAD patients.

Variable	CAD control group	CAD cases group	p
Anthropometric data			
Age, years	51.1 ± 10.3	57.0 ± 10.2	0.005
Gender, male/female	(41/61)	(97/65)	0.005
BMI, kg/m^2	25.5 ± 3.2	26.3 ± 4.1	0.3
Physical inactivity, % (n)	52.9 (54)	64.8 (105)	0.09
Smoking, % (n)	5.9 (6)	20.0 (32)	0.005
Diabetes mellitus, % (n)	12.7 (13)	37.7 (61)	0.0001
Hypertension, % (n)	28.4 (29)	41.4 (67)	0.05
FH of premature CAD, % (n)	27.5 (28)	31.5 (51)	0.7
PMH of diabetes mellitus, % (n)	13.7 (14)	32.1 (52)	0.001
Drugs			
Antilipidemic, % (n)	15.7 (16)	25.9 (42)	0.09
Hypoglycemic, % (n)	4.9 (5)	27.8 (45)	0.0001
Nitrates, % (n)	33.3 (34)	52.5 (85)	0.005
β-Blocker, % (n)	56.9 (58)	55.6 (90)	0.9
Calcium antagonists, % (n)	69.0 (7)	14.2 (23)	0.09
ACE inhibitors, % (n)	7.8 (8)	12.3 (20)	0.3
Diuretics	2.0 (2)	2.5 (4)	0.8
Serum (apo)lipoprotein profile			
ApoB, g/L	1.19 ± 0.24	1.36 ± 0.31	0.0001
ApoAI, g/L	1.37 ± 0.19	1.32 ± 0.22	0.04
ApoB/apoAI	0.87 ± 0.18	1.07 ± 0.32	0.0001
Lp(a), g/L	0.34 (0.04–2.10)	0.52 (0.04–2.90)	0.0001*
LDL-C/apoB, mmol/g	2.63 ± 0.65	2.36 ± 0.84	0.01
HDL-C/apoAI, mmol/g	0.80 ± 0.21	0.78 ± 0.20	0.4
Electrophoresis data			
Chylomicron, %	0.1 ± 0.02	0.2 ± 0.6	0.12
β-Lipoproteins, %	50.3 ± 9.9	51.2 ± 10.1	0.62
Pre-β-lipoproteins, %	26.9 ± 12.1	29.1 ± 11.5	0.31
α-Lipoproteins, %	22.7 ± 6.8	19.8 ± 5.8	0.008
β/α-lipoproteins	2.4 ± 0.8	2.8 ± 0.9	0.007
Serum lipids profile			
Triglycerides, mmol/L	1.80 (0.53–8.13)	2.14 (0.69–16.11)	0.005*
Total cholesterol, mmol/L	5.02 ± 1.06	5.32 ± 1.57	0.07
HDL-C, mmol/L	1.11 ± 0.34	1.02 ± 0.29	0.03
LDL-C, mmol/L	3.10 ± 0.87	3.34 ± 1.53	0.20
LDL-C/HDL-C	3.19 ± 1.48	3.52 ± 2.12	0.15
Non-HDL-cholesterol, mmol/L	4.00 ± 1.14	4.32 ± 1.60	0.07
Total/HDL-cholesterol	4.99 ± 1.83	5.57 ± 2.31	0.04

The number of subjects in each group is shown in parentheses. The factors 0.0113 and 0.0259 were used to convert the concentrations of triglyceride and cholesterol from mg/dL to mmol/L, respectively. ACE, angiotensin-converting enzyme; BMI, body mass index; FH, family history; PMH, past medical history. *Significant difference by Mann-Whitney test.

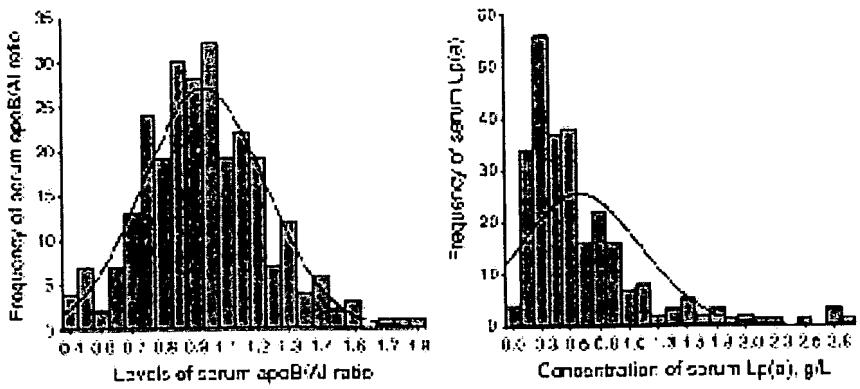

Figure 1 Frequency distribution of apoB/AI ratio and Lp(a) concentrations in the total population. Normal curves are also displayed on the charts.

considered significant if p-values were ≤ 0.05. Both univariate and multivariate logistic regression analyses were carried out to determine the association of variables with the prevalence of CAD (SPSS version 10, SPSS Inc., Chicago, IL, USA). The results of multivariate analysis are expressed as the odds ratio with 95% confidence intervals. A receiver operating characteristic (ROC) curve was used to evaluate the sensitivity and specificity of the tests for CAD using SPSS software.

Results

Anthropometric and biochemical characteristics

The prevalence of diabetes mellitus, hypertension, cigarette smoking and physical inactivity was greater in CAD cases than control subjects (Table 1). There were no significant differences in consumption of hypolipidemic agents, β-adrenergic antagonists, angiotensin-converting enzyme inhibitors, calcium channel blockers and nitrates between the two groups. However, a past history of diabetes mellitus and hypoglycemic medication was more common in patients than in controls.

Patients with CAD had increased serum levels of triglycerides, apoB, Lp(a), and decreased serum levels of HDL-C and apoAI compared with controls. The ratios apoB/apoAI and LDL-C/apoB were also significantly different. Electrophoresis data for serum lipoproteins showed significant lower levels of α-lipoproteins in CAD patients, which is in accordance with the chemical data.

Frequency distributions of apoB/apoAI ratio, apoB and Lp(a)

The frequency distributions of the apoB/apoAI ratio and apoB concentrations were normal, with the mean levels of 0.98 ± 0.29 and 1.29 ± 0.30 g/L, respectively (Figure 1). All other biochemical variables also exhibited a normal Gaussian distribution, except for Lp(a), triglycerides and glucose. Lp(a) concentrations were highly skewed to lower values, with a mean of 0.58 ± 0.52 g/L and median of 0.44 (0.04–2.90) g/L. The mean and median Lp(a) values were 0.05–0.10 g/L higher in females than in males in both the control and patient groups.

Multivariate logistic regression analysis

Multiple logistic regression analysis with a conditional forward approach was performed between control and CAD subjects using CAD as the dependent variable (Table 2). The criteria for inclusion and removal of the variables from the regression equation were 0.05 and 0.1, respectively. The biochemical parameters were entered in the form of continuous variables and the odds ratios were presented as the standardized regression coefficients by the term Exp(β) (associated with 1 SD changes in the risk factor). All anthropometric, physiological and biochemical variables were entered; however, only diabetes mellitus, male sex, apoB, age, Lp(a), hypertension and cholesterol were significant for inclusion in seven subsequent models. If both apoB/apoAI ratio and

Table 2 Multivariate logistic regression analysis.

Model	Variables included	R^2	OR Exp(β)	95% CI	Classified correctly, %	p
1	Diabetes mellitus	0.090	5.92	2.36–14.87	64.5	0.0001
2	+Sex	0.197	6.06	2.79–13.18	77.2	0.0001
3	+ApoB/apoAI	0.252	28.68	4.49–183.25	78.5	0.0001
4	+Age	0.295	1.06	1.02–1.10	78.5	0.001
5	+Lp(a)	0.324	1.02	1.01–1.03	79.4	0.001
6	+Hypertension	0.345	2.36	1.08–5.14	79.8	0.03
7	+Cholesterol	0.359	1.01	1.00–1.02	81.6	0.05

In each model a new variable was added to the previous variables, and the data of the last model with seven parameters is presented. R, multiple correlation coefficient; OR, odds ratio; CI, confidence interval.

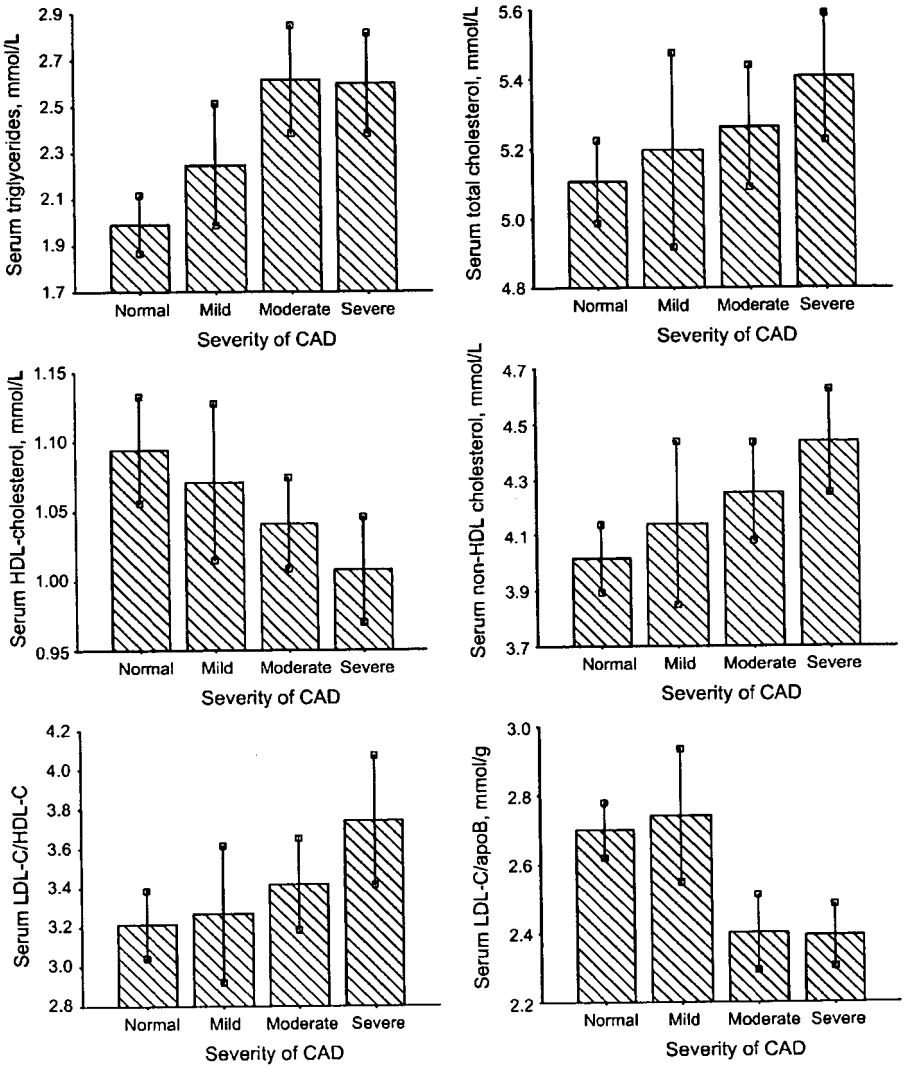

Figure 2 Association of serum triglycerides and cholesterol fractions/ratios relative to the severity of CAD. The severity of CAD was scored on the basis of the number and extent of lesions in coronary arteries as normal, mild, moderate or severe. The value of each variable in any group was calculated and presented as the mean ± SD.

apoB were entered in the analysis, the former was the better predictor. When the results were not adjusted for physical inactivity, triglyceride was also a significant predictor of CAD (OR = 1.004, CI 1.001 – 1.006, $p \leq 0.005$). The efficiency of the tests in each model, i.e., the percentage of subjects correctly classified as CAD controls or cases, was 64.5% for the first model and improved to 81.6% in the seventh model.

Association of serum lipids and (apo)lipoproteins with the severity of CAD

Figures 2 and 3 show that the levels of serum apoB/apoAI ratio [$F(3,264) = 8.6$, $p \leq 0.0001$], apoB [$F(3,264) = 8.5$, $p \leq 0.0001$], Lp(a) [$F(3,264) = 8.6$, $p \leq 0.0001$] and triglycerides [$F(3,264) = 2.3$, $p \leq 0.06$] exhibited significant association, and of total cholesterol [$F(3,261) = 0.5$, $p \leq 0.6$], LDL-C [$F(3,238) = 0.7$, $p \leq 0.6$], HDL-C [$F(3,240) = 1.0$, $p \leq 0.4$] and apoAI [$F(3,262) = 1.3$, $p \leq 0.3$] showed modest association with the severity of CAD. There was no sex difference for the correlation of Lp(a) with the severity of CAD. The results also indicate that LDL-C and non-HDL-C showed no preference relative to total cholesterol for the association with the severity of CAD.

ROC analysis

ROC analysis was used to evaluate the efficiency of the tests for CAD. The ROC curve can be constructed by plotting sensitivity vs. 1–minus specificity or the true-positive rate vs. the false-positive rate. The results are presented in Table 3, and Figure 4 shows the ROC curve for the apoB/apoAI ratio. The area under the ROC curve (AUC) as a relative measure of test efficiency was highest and significant for the apoB/apoAI ratio, apoB, Lp(a), LDL-C/apoB ratio, triglycerides and apoAI.

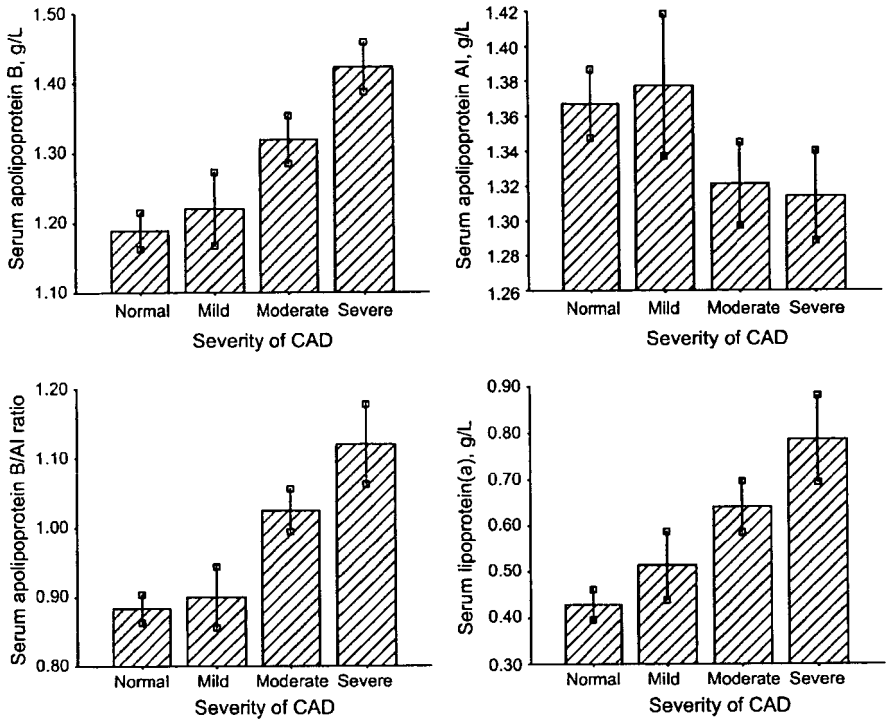

Figure 3 Association of serum (apo)lipoproteins with the severity of CAD.

Discussion

Serum triglycerides, lipid triad and CAD

The results of the present study show that serum triglyceride was associated with the prevalence and severity of CAD and had high diagnostic value for CAD. Furthermore, serum triglyceride was an independent risk factor in univariate analysis, but the association lessened in multivariate analysis. The role of hypertriglyceridemia in the development of CVD is uncertain (24, 25). Growing evidence provided by some prospective studies supports the hypothesis that serum triglyceride is correlated to increased risk of CAD (26, 27). However, not all hypertriglyceridemic patients are at risk for CVD (24). The strength of triglyceride levels in predicting CAD lies in the ability to reflect the presence of atherogenic triglyceride-rich lipoprotein remnants (28). In general, hypertriglyceridemia combined with elevated LDL-C (or small dense LDL) and decreased HDL-C, a pattern known as lipid triad, increases the risk of CAD. Currently, it is suggested that hypercholestrolemia directly causes atherosclerosis, whereas hypertriglyceridemia is better viewed as a marker for increased risk, instead of an independent risk factor for CAD (29).

Table 3 ROC curve analysis for the profile of serum lipids and (apo)lipoproteins.

Variable	Area under curve	p-Value
ApoB/apoAI	0.71±0.03	0.0001
ApoB	0.67±0.04	0.0001
Lp(a)	0.63±0.04	0.001
LDL-C/apoB	0.62±0.04	0.005
Triglyceride	0.62±0.04	0.004
ApoAI	0.58±0.04	0.05
Total/HDL-C	0.56±0.04	0.1
Total cholesterol	0.54±0.04	0.32
HDL-C	0.54±0.04	0.20
Non-HDL-C	0.54±0.04	0.3
HDL-C/apoAI	0.53±0.04	0.50
LDL-C/HDL-C	0.53±0.04	0.55
LDL-C	0.52±0.04	0.76

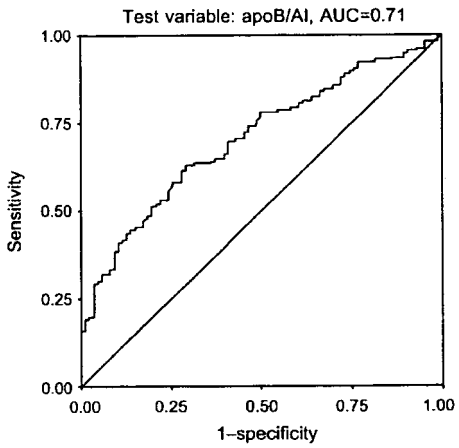

Figure 4 ROC curve for serum apoB/apoAI ratio. Data for all subjects were used to plot the curve. An area under the ROC curve (AUC) of 1.0 indicates perfect discrimination, whereas an area of 0.5 (straight line) indicates that the test discriminates no better than chance.

Cholesterol subfractions and ratios and CAD

The role of total cholesterol and its subfractions in atherogenesis is well documented (30, 31). The results presented here reveal that differences in serum total, non-HDL-C and LDL-C were modest and in HDL-C were significant between CAD control and patient groups. The results were also confirmed by electrophoresis data. This pattern might be due to the high frequency of use of various drugs that can influence serum lipids in CAD patients, as reported for β-blockers (3, 7). Such patterns in total cholesterol and its subfractions have also been reported by others (6, 23, 31). Our results also show that there were no preferences between total, non-HDL-C and LDL-C in predicting CAD.

It has also been shown that LDL is structurally heterogeneous on the basis of its size and density (32). LDL particles have different powers of atherogenicity and smaller, denser particles are more atherogenic than others (32–34). The decreased LDL-C/apoB ratio is thought to be an indirect marker of the small and dense LDL subclass pattern and has been linked to the risk of CAD (32). Our CAD patients had lower values for the LDL-C/apoB ratio, implying compositional changes in LDL particles. The results of ROC analysis demonstrated that the LDL-C/apoB ratio was more efficient than total cholesterol and its subfractions for discriminating CAD patients from control individuals.

Serum apoB, apoAI and apoB/apoAI ratio and CAD

Both logistic regression and ROC analyses of our data confirmed that the apoB/apoAI ratio and apoB had the highest efficiencies for predicting stable CAD. The results are consistent with most of the cross-sectional (3–7) and prospective studies (8–11). However, contradictory results have also been reported (35, 36). On the other hand, apoAI, the major apoprotein of HDL, has a central role in antiatherogenic properties. Therefore, the apoB/apoAI and LDL-C/HDL-C ratios represent the balance between proatherogenic and antiatherogenic (apo)lipoproteins. There is evidence to strengthen the idea that the protein relative to the lipid portion of lipoproteins is a better index for risk assessment (5–11). First, each apoB-containing lipoprotein has only one molecule of apoB per particle. Thus, serum apoB levels reflect the total number of potentially atherogenic lipoprotein particles. However, the levels of serum LDL-C do not adequately represent the number of particles, owing to variations in composition and size. It has been shown that the likelihood that an atherogenic particle is in contact the endothelium, and enters and is trapped within the arterial wall is directly related to the number and inversely related to the size, instead of the cholesterol content of the particles (9). Second, direct measurement of apolipoproteins has been standardized, but LDL-C is estimated on the basis of calculation and has some limitations (10). Moreover, factors such as hospitalization, lipid-lowering diets commonly adopted by CAD patients, and use of β-blockers and antilipidemic drugs can influence lipid parameters, although they have little effect on apoB levels, resulting in the finding that apoB is a much more significant factor associated with CAD (3).

Serum Lp(a) and CAD

The current results indicate a significant difference in serum Lp(a) in subsets of patients with different severity of CAD. Lp(a) was also an independent risk factor and had high predictive value for stable CAD. The association of Lp(a) with the development of CAD has been confirmed by most cross-sectional (6, 7, 23) and prospective studies (13–15). Nevertheless, some studies have failed to identify Lp(a) as an independent risk factor for CAD (5, 16–19). The relation seems to be population- or race-dependent, and differences in Lp(a) assay may also account for these discrepancies (3). Some reports also indicate that Lp(a) is not as strong a risk factor for CAD in women as in men (16, 18). However, analysis of the present data, in accordance with others, showed no sex dependence (15, 37, 38).

Conclusions

The results of the current study indicate significant differences in serum (apo)lipoproteins between subsets of patients with different severity of CAD. The apoB/apoAI ratio, apoB and Lp(a) were also independent risk factors and had high predictive values for stable CAD. The data also demonstrate that the apoB/apoAI ratio is a more powerful index than the conventional cholesterol ratios and provide further evidence that the ratio could be adopted as the best test to identify subjects at risk for CAD in clinical practice.

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Exhibit I

The Survivin:Fas Ratio Is Predictive of Recurrent Disease in Neuroblastoma

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Background/Purpose: Several clinical and biologic features of neuroblastoma (NB) are used to predict the risk of recurrent disease. The balance between antiapoptotic and proapoptotic factors within a tumor may affect its ability to survive. Survivin is an antiapoptotic factor expressed in highly proliferative NB, whereas Fas is a proapoptotic factor that portends a favorable prognosis. The authors determined whether the ratio of survivin to Fas (S:F ratio) is predictive of recurrent disease in patients with NB. The authors previously have shown the S:F ratio is predictive of recurrent disease in pediatric renal tumors.

Methods: The authors quantified the levels of 9 different apoptotic mRNA species using Rnase Protection assay (RPA, Riboquant, PharMingen, San Diego, CA). Twenty-eight primary tumor specimens were evaluated from patients with ganglioneuroma (n = 3), ganglioneuroblastoma (n = 2), and neuroblastoma (n = 23) from tumors of all clinical stages obtained at the time of diagnosis. mRNA levels were calculated as a percentage of L32 for each specimen assayed, and positive expression was assumed to be greater than 10% of L32.

Results: Survivin was expressed in 90% of tumors that went on to recur and only in 27.7% of those that were cured. The S:F ratio was significantly greater in tumors that went on to recur (n = 10) compared with those from patients that were cured (n = 18) (median S:F ratio, 3.3 *v* 0.75; $P = .0002$, Wilcoxon rank-sum test). A cutoff ratio of 2.3 was highly predictive of tumor recurrence irrespective of clinical stage of disease (area under ROC curve = 0.906). Sensitivty was 80% (CI, 44.4% to 97.5%), specificty was 94.4% (CI, 72.7% to 99.9%), positive predictive value was 88.9% (CI, 51.8% to 99.7%), and negative predictive value was 89.5% (66.9% to 98.7%). Twenty-five of 28 (89.3%) tumor ratios were correct in predicting outcome.

Conclusions: The survivin:Fas ratio in primary tumors may be used to predict the risk for recurrent disease in patients with NB. The S:F ratio appears to be a more sensitive predictor of recurrent disease than survivin expression alone. Determining this ratio may not only be helpful in guiding follow-up of patients with NB, but also may aid in stratifying patients for more aggressive therapeutic strategies.

INDEX WORDS: Neuroblastoma, apoptosis, Fas, survivin, prognosis.

NEUROBLASTOMA (NB) is a malignant tumor of childhood that arises from primordial neural crest cells. This tumor accounts for 8% to 10% of all childhood cancers and for 15% of cancer related deaths in children.[1] NB is distinct in its ability to "spontaneously" regress, but more frequently presents as a markedly aggressive tumor with a high risk for recurrence. Several clinical, pathologic, and biologic features of NB are used to predict the risk of recurrent disease. Many of the biologic markers used to predict prognosis potentially reflect the tumor's ability to proliferate (N-*myc*, PCNA)[2,3] or differentiate (NGF, TRK-A, Somatostatin receptors).[4-6]

Apoptosis, or programmed cell death is the process by which activated cells undergo a suicide program that results in individual cell death.[7] It is a highly orchestrated process in which cells die in a regulated fashion. Although apoptotic factors are thought to contribute to the pathogenesis of certain tumors, the exact role of apoptosis in oncogenicity is not clearly defined. It is feasible that the balance between antiapoptotic factors and proapoptotic factors within a tumor cell could affect the cell's survival.

An abundance of apoptosis inhibitors could protect dividing cells from dying, thus, contribute to tumorigenicity. A related family of inhibitors of apoptosis proteins (IAP) is described in many species, and these proteins block apoptosis by direct inhibition of downstream effector caspases in the pathway of cell death.[8,9] A novel member of the inhibitor of apoptosis family of genes, known as survivin, is detected in many tumors but is absent in most normal differentiated adult tissues.[10] Survivin is expressed during cell proliferation in the G2/M phase of the cell cycle, and its presence in tumors of patients with neuroblastoma, pediatric renal tumors,

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and bladder and gastric cancer portends a poor survival.[11-14] Survivin plays an important role in cell cleavage and appears to block default induction of apoptosis during mitosis. Survivin thus acts at the interface between apoptosis and the cell cycle and when overexpressed in cancer cells, it may preserve cell viability during cell division.[15,16]

Although there are multiple agents that can induce apoptosis, perhaps the most characterized are the death-inducing members of the tumor necrosis factor (TNF) family of cytokines and their cognate receptors.[17] For example, TNF and Fas ligand (FasL) function as apoptotic mediators in many physiologic events, including autoimmunity and activation-induced cell death by binding to their receptors.[18,19] TNF and FasL also are extremely efficient at killing a variety of tumor cells; however, the proapoptotic receptor Fas is found to be absent in pediatric renal tumors[20] and neuroblastoma[21] of poor prognosis. In a previous study, we found that the ratio between survivin (antiapoptotic) and Fas (proapoptotic) was predictive of recurrent disease in pediatric renal tumors irrespective of stage or pathologic features.[12] This study aims to evaluate the power of the survivin to Fas (S:F) ratio in predicting the risk of recurrent disease in patients with neuroblastoma.

MATERIALS AND METHODS

Tissue Specimens

Twenty-eight frozen tissue specimens were obtained from pediatric patients with NB and related tumors before chemotherapy at the University of Iowa Hospitals and Clinics and from the Cooperative Human Tissue Network (CHTN) tissue bank. Specimens were as follows: NB (n = 23; Evans stage I, n = 4; stage II, n = 2; stage III, n = 6; stage IV, n = 8; stage IVs, n = 3); ganglioneuroblastoma (GNB, n = 2); and ganglioneuroma (GN, n = 3). Our Institutional Review Board approved all protocols and tissues harvested.

RNase Protection Assay

The RiboQuant Multiprobe protection assay system (PharMingen, San Diego, CA) is a highly sensitive and specific method for simultaneous detection and quantification of multiple mRNA species. Human apoptosis template probe sets are available, and we have customized a template (Custom human template set; PharMingen) to detect levels of XIAP, survivin, Fas, DCR1, DR3, DR5, DR4, Bax, tumor necrosis factor (TNF)-R1, L32, and GAPDH. These mRNA species were selected from previous studies in which we examined mRNA species in pediatric renal tumors,[12] and among the panel selected are those mRNA species that appeared to be differentially present in tumors.

After isolating RNA from the tissues of interest, the antisense RNA probe was hybridized in excess to target RNA in solution. Free probe and nonhybridized single-stranded RNA were digested with RNases. ^{32}P-labeled antisense RNA was transcribed using T7 RNA polymerase (PharMingen) and [α-^{32}P]UTP (Amersham Life Science, Piscataway, NJ). Total RNA was extracted from tissue using TRIZOL (Gibco Life Technologies, Carlsbad, CA). Fourteen to 16 μg of total RNA was hybridized with the ^{32}P-labeled RNA probes that were transcribed from the customized multiprobes (DNA templates) at 56°C overnight, followed by digestion with a 1:417 dilution of RNase "cocktail" (RNase A:T1 mixture; PharMingen) for 45 minutes at 30°C. Single-stranded and unhybridized excess mRNA was digested by the RNase "cocktail". The protected double-stranded RNA pellets were dried and resuspended in 5 μL of 1 x loading buffer (PharMingen), and electrophoretically resolved on 5% polyacrylamide-8 M urea gel. The polyacrylamide-urea gel was dried on blotting paper for 1 hour at 80°C. The labeled probes then were quantified by autoradiography by exposing the blotting paper to Bio Max Film (Kodak, Rochester, NY) with an intensifying screen (Kodak) for 20 hours at −80°C.

Radioanalytic Imaging

For comparative analysis, we quantified mRNA levels as a percentage of the ribosomal protein L32 mRNA level. Radioactivity of each band in the sample was quantified, standardized, and compared with the level of L32 detected in that sample. Radioactivity of each template was quantified directly from the gel by a radioanalytic imaging system (AMBIS 4000, AMBIS Inc, San Diego, CA) equipped with AMBIS QuantProbe Software Version 3.0 for 1,000 minutes. Net counts were obtained from each template (band) including L32, and the radioactivity was expressed as a percent of the L32 activity.

Data Analysis

Groups were divided into tumors that went on to recur and tumors that did not recur. Survivin:Fas ratios were determined and compared among the groups of tissue specimens using the Wilcoxon rank-sum test. Because we found a significant association between a high survivin:Fas ratio and recurrence of tumor, the survivin:Fas ratio can be used as a predictor of recurrent disease. Sensitivity, specificity, and positive and negative predictive values of this ratio were computed. The median, mean, and standard deviations are presented.

RESULTS

Outcomes

We examined 28 primary (pretreatment) tissue specimens, of which, 10 tumors went on to recur, and 18 were cured after therapy. Nine of the ten patients with recurrent disease have died of their disease, whereas the remaining patient has active disease and is failing treatment. The group of tumors that went on to recur included NB (stage III, n = 3 and stage IV, n = 7). The group of tumors that were cured included: NB (stage I, n = 4; stage II, n = 2; stage III, n = 3; stage IV, n = 1; and stage IVs, n = 3); GNB (n = 2), and GN (n = 3).

Assuming positive survivin expression as an mRNA level greater than 10% of L32, 90% of tumors that went on to recur expressed survivin, whereas only 27.7% of tumors that were cured were positive for survivin. Seventy-one percent of advanced-stage tumors (III and IV) were positive for survivin. Ten percent of L32 mRNA expression was chosen empirically to be positive from correlative studies of protein expression in pediatric renal tumors[12] and compares favorably with reported immunocytochemical studies in NB.[11]

The survivin:Fas ratio was calculated from RPA values (Fig 1), and this mRNA ratio was significantly greater in the group of tumors that went on to recur than in nonrecurrent tumors (P = .0002 Wilcoxon rank-sum test). The median survivin:Fas ratio in the recurrent

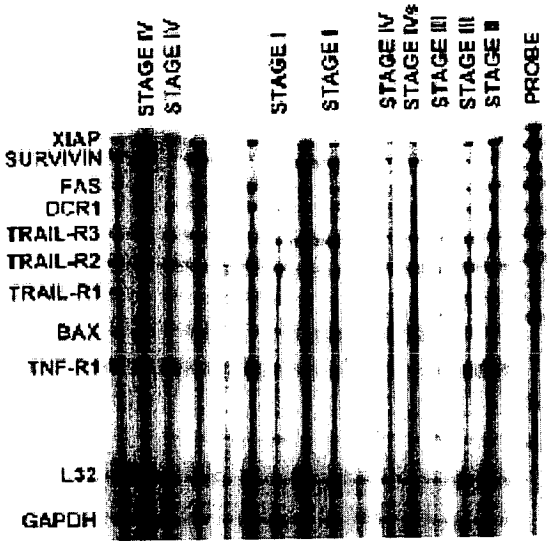

Fig 1. Example of an autoradiographic plate of RNase protection assay (RPA) shows bands marking the various mRNA species detected in several NB tumors. Unlabeled lanes are bands from tumors not used in this study. Each mRNA band of interest is expressed as a percent of L32 (Ribosomal mRNA).

group was 3.30 (interquartile range, 2.5 to 2.0). The median survivin:Fas ratio in the nonrecurrent disease group was 0.75 (interquartile range, 0.4 to 1.58). Eight of 10 tumors that went on to recur had a survivin:Fas ratio of 2.5 or greater, whereas 2 of the 18 tumors that were cured had a ratio of 2.3 or greater. Using a survivin:Fas ratio of 2.3 as cutoff, the sensitivity of this test was 80% (confidence interval [CI], 44.4%, 97.5%) with a specificity of 94.4% (CI, 72.7%, 99.9%). The positive predictive value of this ratio was 88.9% (CI, 51.8%, 99.7%) and the negative predictive value was 89.5% (CI, 66.9%, 98.7%). The survivin:Fas tumor ratio was correct in predicting recurrence of disease in 89.3% (25 of 28) of cases (Table 1).

The receiver operating characteristic (ROC) curve was constructed and the area under the ROC curve was 0.9 ± 0.06. The area under the ROC curve represents the probability that a randomly selected pair of patients, one with recurrence and one without recurrence, will both be classified correctly by the test.

DISCUSSION

Apoptosis is considered an important process in the biological behavior of tumors.[22] An abundance of factors preventing cell death, or a depletion of factors promoting cell death may affect tumor survival. In the current study, we show that abundant expression of the antiapoptotic factor survivin and the absence of the proapoptotic factor Fas, appears to be associated with recurrence of NB. When the ratio of expression between these 2 apoptotic factors is determined in untreated primary NB specimens, it is a sensitive and specific test for predicting tumor recurrence.

N-*myc* amplification currently is the gold standard of molecular markers used for predicting NB risk for recurrence but is only applicable in about 40% of cases.[23] It is associated with advanced stage of disease, rapid tumor progression, and poor prognosis. How N-*myc* amplification and survivin:Fas ratios compare and relate to each other is unknown, because data on N-*myc* expression was only available in 16 of our 28 patients. In the group of 10 patients whose tumors went on to recur, N-*myc* data was available in 6, of which, it was amplified in 4 of the tumors. Similarly, 5 of these 6 tumors had S:F ratios greater than 2.5. It is of interest to note that only about 40% of advanced-stage tumors express N-*myc*,[24] whereas in our small series, 78.5% (n = 14) of tumors of advanced primary stage (stage III and IV) expressed survivin and 80% of the tumors that went on to recur had S:F ratios greater than 2.5.

Survivin is expressed in multiple tumors and is associated with both unfavorable histology and higher stage of disease.[11-14] Survivin is a member of the family of Inhibitors of Apoptosis proteins and is the only antiapoptotic factor described thus far that appears to be cell cycle dependent. Survivin is expressed in the G2/M (mitosis) phase of the cell cycle and is localized to mitotic spindle microtubules.[16] Others have shown that survivin is associated with mitotic kinetochores when microtubule assembly is disrupted and its localization is, thus, independent of microtubules.[25] Nevertheless, a consensus of data suggests that survivin is positioned to have an important function in maintaining cell viability during mitosis, potentially coupling apoptosis control to the mechanism of cell cleavage. When overexpressed in cancer cells, survivin may not only secure aberrant proliferation through mitosis, but also probably enhances this process. It is of interest to note that survivin is absent in most differentiated mature tissues, whereas it is present in developing fetal tissue and mitotically active tumor.

Survivin acts as a mitotic substrate of the cyclin-dependent kinase $p34^{cdc2}$-cyclin B1, and phosphorylation of survivin may regulate apoptosis at cell division by

Table 1. Survivin:Fas Ratio as a Predictor of Neuroblastoma Recurrence

Cut-off Value S:F Ratio	Recurrent Tumors	Nonrecurrent Tumors
<2.32	2	16
≥2.32	8	2
Sensitivity	80%	(CI: 44.4%-97.5%)
Specificity	94.4%	(CI: 72.7%-99.9%)
Positive predictive value	88.9%	(CI: 51.8%-99.7%)
Negative predictive value	89.5%	(CI: 66.9%-98.7%)

neutralizing caspase-9.[26] Caspase-9 is part of an evolutionary conserved "apoptosome" complex that induces down-stream activation of effector caspase in the mitochondrial pathway of apoptosis. Dissociation of a survivin-caspase-9 complex on the mitotic apparatus, led to caspase-9–dependent apoptosis of cells traversing mitosis.[26] These observations suggest that survivin plays an important role in cell proliferation and, thus, its expression in tumors of higher grade and worse prognosis would compliment its proposed function as an antiapoptotic protein at the interface between apoptosis and tumor cell division.

Unlike survivin, Fas is a proapoptotic factor and is a member of the TNF-related family of "death receptors".[17] Expression of Fas by tumors of good prognosis may implicate tumor cell destruction by immune effector cells as a means of controlling disease. Relating prognosis to expression of proapoptotic (TNF family) receptors is an appealing concept, because both immune surveillance cytotoxic T-cells and Natural Killer cells use these receptor pathways during target cell destruction. Survivin is shown to inhibit apoptosis induced by several ligands of the TNF-related family of receptors; thus enhancing cell survival and preventing cell death.[27] Although current literature suggests that the functions of survivin and Fas are potentially dependent on one another, it also is possible that these 2 factors act independently in regulating different apoptotic pathways.

We propose that the quantified survivin:Fas mRNA ratio in primary NB tissue may be used to identify patients at high risk for recurrent disease. We believe that this ratio is biologically feasible, in that the balance between pro- and antiapoptotic factors could be crucial to a tumor's survival. Almost 30% of tumors that were eradicated in this study expressed survivin. Thus, determining the ratio of survivin to fas appears to improve the positive predictive value when compared with an evaluation of survivin expression alone. This ratio may not only be helpful in guiding follow-up of patients with neuroblastoma, but also may aid in stratifying patients for more aggressive initial therapeutic strategies. Whether this biologic molecular ratio is applicable to all tumor types is conjectural, but previous studies from our laboratory show that the S:F ratio is also predictive of recurrent disease in pediatric renal tumors.[12]

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Discussion

M. LaQuaglia (New York, NY): Tony, it is interesting work. I was interested in whether you looked at Fas or Fas ligand and saw what the absolute value of that was. We have seen data in which Fas and Fas ligand can be upregulated resulting in apoptosis, but it has not involved survivin.

The other question I had is just philosophical. With all the prognostic factors that are available for neuroblastoma, how does this fit in in a multivariate scheme?

A. Sandler (response): As far as Fas and Fas ligand goes, Fas is not expressed frequently in neuroblastoma. Fas ligand has not been detected in our cell lines or tumors studied. In this study, because Fas was more frequently expressed in the "good prognostic tumors," we thought it would add to survivin's ability to be predictive of recurrent disease. Fas alone appears to be infrequently expressed in neuroblastoma and, thus, alone would not be predictive of recurrent disease.

As to your second question: for neuroblastoma we have a very good marker, N-*myc*, but it is only present in about 40% of late-stage disease. It is of great prognostic value but is not good for the majority of these tumors. We believe that the survivin-Fas ratio is very predictive. I do not know if this ratio is useful in all tumors. Last year we showed it was predictive for pediatric renal tumors. Currently, we are doing a blinded study with the National Wilms' Tumor Study Group in which we evaluated 80 tissue specimens and will see how those turn out. Time will tell whether it is more predictive than N-*myc* for neuroblastoma.

M. LaQuaglia: It's not just N-*myc*. It is ploidy, it is 1P36, it is pathologic stage and . . .

A. Sandler: Absolutely, and I think this will either add to the multivariate analysis or potentially be used alone.

Exhibit J

disease manifest

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Serum C-reactive protein is an independent risk factor predicting cardiometabolic risk

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Abstract

The aim of the study was to investigate the role of serum C-reactive protein (CRP) level as a risk factor in predicting metabolic syndrome (MS), hypertension, atherogenic dyslipidemia, type 2 diabetes mellitus, and coronary heart disease. We prospectively evaluated 1270 men and 1320 women, aged 30 to 89 years, who had serum CRP determinations and a mean 4.3 years' follow-up. The CRP values were log-transformed for calculations. Metabolic syndrome was defined by the Adult Treatment Panel III criteria modified for male abdominal obesity. Prediction of outcome was performed by excluding from analysis the particular outcome variable existing at baseline examination. Smoking men had higher age-adjusted estimated CRP concentrations ($P < .001$), whereas smoking women had lower CRP ($P = .027$) than never smokers. Risk of developing an elevated (≥2 mg/L) CRP was predicted significantly by baseline CRP in both sexes and by apolipoprotein (apo B), current smoking, and family income in men, when adjusted for 5 further variables. Baseline CRP levels predicted atherogenic dyslipidemia when adjusted for age, baseline dyslipidemia values, and apo B tertiles and predicted incident hypertension independent of age, waist circumference, and smoking status. After adjustment for sex, age, and the 5 MS components, CRP predicted newly developing MS, with a hazard ratio (HR) of 1.16 (95% confidence interval, 1.02-1.32). When adjusted for sex, age, baseline glucose, waist circumference, and apo B tertiles, diabetes was significantly predicted by CRP in women (HR, 1.31) alone. Sex- and age-adjusted CRP level identified also those that progressed to diabetes independent of a fasting glucose >100 mg/dL (HR, 1.39; 95% confidence interval, 1.21-1.59), although not in men. In the prediction of incident coronary heart disease, CRP contributed to 7 established risk factors including waist circumference with a significant 1.18-fold HR. C-reactive protein is both an independent significant predictor and a risk factor of cardiometabolic risk among Turkish adults, additive to MS components, whereby risk is modulated by sex, smoking habit, and apo B.

1. Introduction

Serum concentrations of the acute phase reactant C-reactive protein (CRP) have been recognized in the past decade as a consistent marker of developing coronary heart disease (CHD), thus linking low-grade systemic inflammation with atherosclerosis. Prospective data from epidemiologic studies disclosed a significant relationship between CRP and future CHD risk in apparently healthy men [1-3] and women [4,5]. Elevated levels of CRP offer predictive value exceeding that of low-density lipoprotein (LDL) cholesterol [6]. Nonetheless, absence of a relationship of CRP with risk of myocardial infarction has also been reported in men when comprehensive adjustment was made for established risk factors [7].

Low-grade inflammation has been postulated to be linked also to the development of metabolic disorders; and some prospective epidemiologic evidence has been accumulated regarding diabetes [8,9], hypertension [10-12], and MS [8,9]. However, this may still be considered as scarce. Positive relationships between the risk of developing hypertension and elevated CRP levels were reported recently in men [11] and women [12]. Prospective studies linking inflammation with MS have also been few [8,9,13], especially among women; and a recent study using a mendelian randomization approach suggested that CRP is causally not related to MS in British women [14].

When the predictors of inflammatory markers (interleukin 6, CRP, tumor necrosis factor-α, and others) in 77

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nondiabetic postmenopausal overweight and obese women were investigated, apolipoprotein (apo) B was found to be the primary predictor among a variety of risk parameters (adiposity, blood pressure [BP], insulin resistance, triglycerides, apo B/apo A-I ratio, Framingham risk points, etc) [15]. Among Turkish adults who have a high prevalence of metabolic syndrome (MS) [16], apo B appears to be independently related not only to incident CHD but also to hypertension, MS, and diabetes [17]. Moreover, a diverging effect of current smoking among female and male Turks emerges on certain major metabolic disorders [18], which necessitates the examination of its role in the development of raised CRP and its modifying influence on the CRP-related cardiometabolic risk.

Underscoring the inability to separate hypertension from the injurious agents that initiate arterial inflammation, such as cigarette smoking and agents of metabolic origin, it was pointed out in a recent editorial that research is moving toward the interface between inflammation and metabolic disturbances, which is where arterial disease occurs [19]. It is therefore worth addressing the pathways from low-grade inflammation to metabolic disorders such as atherogenic dyslipidemia, hypertension, MS, and diabetes and to CHD prospectively in a suitable cohort. The present article investigates longitudinally the following issues in a cohort representative of Turkish adults [20]: (*a*) Which risk parameters determine risk for developing an elevated CRP? (*b*) To what extent are CRP levels associated with the development of the stated cardiometabolic risks? (*c*) Finally, do sex, smoking habit, and apo B, another agent with prominent proinflammatory properties, modulate the stated risks? This article discloses novel findings related to the prediction by CRP of atherogenic dyslipidemia, hypertension, and MS and to the modulation of CRP-related risk by sex, female-specific smoking, and apo B.

2. Methods

2.1. Population sample

The Turkish Adult Risk Factor Study is a prospective cohort study on the prevalence of cardiac disease and risk factors in adults in Turkey carried out periodically almost biennially since 1990 in 59 communities scattered throughout all geographical regions of the country [20]. It involves a random sample of the Turkish adult population representatively stratified for sex, age, geographical regions, and rural-urban distribution [20]. Measurements of CRP were first performed at the survey of 2000, which formed the baseline. Participants were 30 years of age or older. Of the survivors, 7% were examined up to the survey of 2001-2002 and 12% up to 2003-2004, the remainder having been examined lastly in the survey of 2005-2006. Serum CRP was measured in 2709 men and women at baseline. Exclusion of 5 individuals having age >89 years and 114 persons with CRP values >15 mg/L (given that extreme CRP values are usually not associated with cardiometabolic disorders) limited the study sample to 2590 adults (1270 men and 1320 women). Nearly 90% of baseline participants of the present and the previously reported prospective study [17] overlapped. The survey conformed to the principles embodied in the Declaration of Helsinki and was approved by the Istanbul University Ethics Committee. Individuals of the cohort were visited in their addresses on the eve of the examination and were requested to give written consent for participation after having read an explanatory note, which was manifested by their voluntary participation the next morning. Data were obtained by history of the past years via a questionnaire, physical examination of the cardiovascular system, sampling of blood, and recording of a resting 12-lead electrocardiogram (ECG).

2.2. Measurements of risk variables

A history of infection in the month preceding the survey or of systemic inflammation was not elicited. Blood pressure was measured in the sitting position on the right arm, and the mean of 2 recordings at least 3 minutes apart was recorded. Weight was measured without shoes in light indoor clothes using a scale. Waist circumference was measured with a tape (Roche LI95 63B 00; Roche Diagnostics, Mannheim, Germany), with the subject standing and wearing only underwear, at the level midway between the lower rib margin and the iliac crest. Body mass index was calculated as weight divided by height squared (in kilograms per square meter). Self-reported cigarette smoking status was categorized into nonsmokers, former smokers, and current smokers.

Plasma concentrations of cholesterol, fasting triglycerides, high-density lipoprotein (HDL) cholesterol, and glucose were determined at baseline examination by the enzymatic dry chemistry method using a Reflotron apparatus (Roche Diagnostics). The LDL cholesterol values were computed according to the Friedewald formula. In the final 3 surveys, the stated parameters as well as apo B, insulin, and CRP values were assayed in a single central laboratory. Blood samples were spun at 1000*g* for 10 minutes and shipped within a few hours on cooled gel packs at 2°C to 5°C to Istanbul to be stored in deep freeze at −75°C until analyzed at a central laboratory in the same city. Concentrations of insulin were determined by the chemiluminescent immunometric method using Roche kits and Elecsys 1010 immunautoanalyzer (Roche Diagnostics). Concentrations of serum CRP and apo B were measured by the Behring nephelometry, CRP using an N high-sensitivity CRP kit (Behring Diagnostics, Marburg, Germany) the lower detection limit of which was 0.175 mg/L. Within-run and day-to-day coefficients of variation for CRP were 1.3% and 2.9%, respectively. External quality control was performed with a reference laboratory in a random selection of 5% to 6% of participants. Data on insulin and apo B

were available in two thirds of the participants, and measurements of the studied parameters were available again in their final examination.

2.3. Definitions and outcomes

Individuals with *diabetes* were diagnosed with the criteria of the American Diabetes Association [21], namely, plasma fasting glucose ≥126 mg/dL (or 2-hour postprandial glucose >200 mg/dL) and/or current use of diabetes medication. Individuals with *metabolic syndrome* were identified when 3 out of the 5 criteria of the National Cholesterol Education Program (Adult Treatment Panel III) [22] were met, modified for prediabetes (fasting glucose 100-125 mg/dL) [23] and further for abdominal obesity using as cutpoint ≥95 cm in men, as recently assessed in the Turkish Adult Risk Factor study [24,25]. *Atherogenic dyslipidemia* (or simply *dyslipidemia*) referred to the combined presence of high triglyceride (≥150 mg/dL) and low HDL cholesterol (<40/<50 mg/dL) values as defined by the Adult Treatment Panel III. *Hypertension* was defined as a BP ≥140 mm Hg and/or ≥90 mm Hg, and/or use of antihypertensive medication. Missing data on triglycerides in one eighth of the sample did not preclude the identification of MS because availability of no more than 3 criteria was required, and the MS and/or dyslipidemia status of the subsequent survey was adopted in few individuals presenting 2 positive criteria. Apolipoprotein B cutoff by 120 and 95 mg/dL yielded 519, 381, and 405 (of 1305) men and women in the top, middle, and bottom brackets (40%, 29%, and 31%). Homeostatic model assessment (HOMA) was calculated with the following formula [26]: insulin (in milli–international units per liter)* glucose (in millimoles per liter)/22.5.

Diagnosis of nonfatal CHD was based on the presence of angina pectoris, a history of myocardial infarction with or without accompanying Minnesota codes of the ECG [27], or a history of myocardial revascularization. Typical angina and, in women, age >45 years were prerequisite for a diagnosis when angina was isolated. The ECG changes of "ischemic type" of greater than minor degree (codes 1.1-2, 4.1-2, 5.1-2, 7.1) were considered as myocardial infarct sequelae or myocardial ischemia, respectively. Cause of death was assigned based on information elicited from first-degree relatives and local health center staff, taking into account preexisting clinical and laboratory findings obtained during the biennial follow-ups. Fatal CHD comprised death from heart failure and fatal coronary event.

2.4. Data analysis

Because of the skewed distribution of concentrations of insulin and CRP, these were log-transformed for calculations. Descriptive parameters were shown as mean ± SD or as age-adjusted estimated mean ± SE and in percentages. Two-sided t tests and Pearson χ^2 tests were used to analyze the differences between means and proportions of other groups; univariate analyses followed by pairwise comparisons were made to detect significance between estimated marginal means in smoking groups. Multiple linear regression analyses were performed with continuous parameters. In the prediction of a dependent variable, the cohort in whom that particular variable existed at baseline examination was excluded from the multivariate analysis. Estimates (and 95% confidence intervals [CIs]) for relative risk (RR) of a dependent variable were obtained by use of logistic regression analysis in models that controlled for potential confounders. Hazard ratios (HRs) were calculated using the given RRs for 1 SD (SD = 2.85-fold concentration of CRP). A value of $P < .05$ on the 2-tailed test was considered statistically significant. Statistical analyses were performed using SPSS 10 for Windows (SPSS, Chicago, IL, no. 9026510).

3. Results

At baseline examination, mean age of 2590 participants was 49.8 ± 12 years; and mean follow-up constituted 4.3 years (total 11 100 person-years). Excluded from prospective analysis of outcomes were cases of prevalent CHD or each metabolic disorder, respectively.

3.1. Stratification of CRP values by sex and smoking status

Table 1 summarizes the baseline risk characteristics of the study sample in men and women, which indicate the presence of wide waist girth, comparatively low total and HDL cholesterol levels, and high levels of apo B relative to LDL cholesterol. Age-adjusted estimated marginal means

Table 1
Risk characteristics at baseline, by sex

	Men (n = 1270)			Women (n = 1320)		
	n	Mean	SD	Mean	SD	P
Age (y)		50.0	12.2	49.6	12	NS
Waist circumference (cm)		94.1	10.9	90.5	12.5	*
Systolic BP (mm Hg)		126.9	21.9	131.6	25.4	*
Diastolic BP (mm Hg)		80.9	13.0	82.4	13.9	**
Total cholesterol (mg/dL)		181	37.2	187.6	40.8	*
HDL cholesterol (mg/dL)		37.2	11.7	44.7	12.7	*
LDL cholesterol (mg/dL)	2285	113.8	31.8	119.4	34.9	*
Fasting triglycerides (mg/dL)	2216	155.3	99.3	132.6	85.1	*
Fasting glucose (mg/dL)	2198	99.0	29.3	100.4	28.0	NS
Apo B (mg/dL)	1768	115.0	36.4	112.8	36.2	NS
Fasting insulin[a] (mIU/L)	1674	7.7	2.1	7.8	2.0	NS
CRP[a] (mg/L)		1.77	2.7	2.03	2.9	*
Current/former smoking (%)		52.7/20.5		18.0/3.6		*

Unspecified n equals to 2590. P values > .2 denoted by NS. NS indicates not significant.

[a] Geometric mean values.

* $P < .001$.

** $P < .005$.

Table 2
Age-adjusted serum CRP estimated marginal mean values (in milligrams per liter) at baseline, by smoking status

	Men			Women		
		Geometric mean	SE		Geometric mean	SE
	1270	1.77		1320	2.04	
Never smokers	340	1.52	1.05	1035	2.08	1.03
Former smokers	260	1.67	1.06	48	2.70**	1.16
Current smokers	670	1.96*	1.04	237	1.75*	1.07

* Different ($P < .03$) from never smokers.
** $P = .085$ from never smokers.

(±SE) among male and female subjects by smoking status are shown in Table 2. Smoking men had higher CRP concentrations ($P < .001$), whereas smoking women had lower CRP ($P = .027$) than never smokers.

3.2. Determinants at baseline of subsequent elevated CRP

Paired values of CRP at baseline and final surveys were available in 1487 participants (57.4%). When 47.7% of individuals who had serum CRP values ≥2.0 mg/L at baseline examination were excluded from analysis, determinants at baseline were sought for the prediction of CRP values ≥2.0 mg/L developing among 777 adults in the follow-up. Fasting insulin and apo B levels were available in just over half the sample. Logistic regression analysis was carried out in a model that comprised age, waist circumference, smoking status, family income, systolic BP, apo B, HDL cholesterol, fasting insulin, and baseline CRP concentrations in 412 subjects (Table 3). Baseline CRP was the significant predictor of subsequently elevated CRP in each sex, whereas current smoking, apo B, and family income were so additionally in men. Current smoking was not a determinant of elevated CRP levels in women. Measure of agreement of self-reported categories of smoking was tested separately in men and women between the baseline and subsequent surveys and between the final and the preceding surveys: κ values regarding female smoking status averaged 0.80 ($P < .001$), compared with 0.76 ($P < .001$) in men.

3.3. Prediction of atherogenic dyslipidemia and hypertension

Of those having serum triglyceride determinations at baseline, 27% who met the criteria of atherogenic dyslipidemia were excluded from analysis. Log CRP adjusted for sex, age, and baseline concentrations of HDL cholesterol and triglycerides was associated with future dyslipidemia developing in 178 subjects of 988 men and women (RR, 2.00; 95% CI, 1.33-3.03) (Table 4). Significance persisted in separate sexes (in 409 men: RR, 2.13; 95% CI, 1.10-4.12; in 579 women: RR, 1.78; 95% CI 1.03-3.06). After additional adjustment for apo B tertiles, CRP retained significance (RR, 1.76; 95% CI, 1.05-2.94) but not in separate sexes.

Out of 2125 nonhypertensive persons at baseline, 786 subjects (37%) developed hypertension in the follow-up. Sex- and age-adjusted RR of CRP for new hypertension was 1.62 (95% CI, 1.31-2.01), which proved similarly significant ($P < .007$) in both sexes: 1.54 in men and 1.70 in women. When waist circumference was included in the regression analysis, RR attenuated to 1.29 but remained significant (95% CI, 1.03-1.62) in combined sexes. Introduction of smoking status marginally increased RR to 1.32 (95% CI, 1.05-1.65) and also RR in men to a significant 1.41 (95% CI, 1.02-1.95).

3.4. Prediction of MS and diabetes by CRP

Among 1090 persons free of MS at baseline, 284 subjects (26%) developed MS in the follow-up. After adjustment for sex, age, and baseline values of all 5 MS components (waist circumference, HDL cholesterol, triglycerides, systolic BP, and glucose) as continuous variables, MS was predicted with an RR of 1.53 (95% CI, 1.06-2.19) by CRP (Table 4). All standard components predicted MS significantly (data not shown). With the addition of apo B tertiles (determined in part of the sample) to the previous regression model, the odds of the association of CRP were unchanged (1.53); but the CI widened (95% CI, 0.98-2.42). It became stronger in men and attenuated in women.

Log CRP, adjusted for sex, age, baseline waist circumference, and fasting glucose, in 2089 nondiabetic individuals was a significant predictor of diabetes developing in 174 subjects (RR, 2.10; 95% CI, 1.38-3.20). Significant prediction was observed not in men (RR, 1.50; $P = .17$) but only in women (RR, 3.05; 95% CI, 1.64-5.70) (Table 4). When apo B tertiles were added to the model, RR in sexes combined attenuated to only 1.70 (95% CI, 1.04-2.78) and persisted to be significant in women (RR, 2.15; 95% CI, 1.05-4.39). Sex- and age-adjusted CRP level also identified progression to diabetes in participants with a fasting glucose level >100 mg/

Table 3
Prediction of CRP value ≥2 mg/L by determinants in subjects free of elevated CRP at baseline

	Men (n = 212)		Women (n = 200)	
	RR	P	RR	P
Age (y)	1.007	NS	0.991	NS
Baseline CRP[a]	**10.0**	**.005**	**23.9**	**.0005**
Waist circumference (cm)	1.027	.114	0.986	NS
Current smokers	**2.25**	**.045**	1.006	NS
Former smokers	1.41	NS	3.11	NS
Apo B (mg/dL)	**1.010**	**.027**	0.994	NS
HDL cholesterol (mg/dL)	1.000	NS	0.984	NS
Systolic BP (mm Hg)	0.995	NS	1.009	NS
Family income (I-IV)	**0.74**	**.047**	0.936	NS
Fasting insulin[a]	**0.81**	NS	2.23	.18

Sixty-seven men and women each developed CRP ≥2 mg/L in follow-up. Model comprised 90 male and 30 female current smokers. Significant relative risks are highlighted in bold.
[a] Log-transformed values.

Table 4
Adjusted RRs in the prediction of various metabolic disorders by baseline serum CRP values[a]

	Model	n	Total		Men		Women	
			RR	95% CI	RR	95% CI	RR	95% CI
Atherogenic dyslipidemia	1a	988	**2.00**	1.33-3.03	**2.13**	1.10-4.12	**1.78**	1.03-3.06
	2	652	**1.76**	1.05-2.94	2.25	0.98-5.18	1.46	0.76-2.86
Hypertension	1b	2125	**1.29**	1.03-1.62	1.32	0.96-1.82	1.30	0.94-1.80
	2	2125	**1.32**	1.05-1.65	**1.41**	1.02-1.95	1.30	0.94-1.80
Type 2 diabetes mellitus	1c	2089	**2.10**	1.38-3.20	1.50	0.84-2.68	**3.05**	1.64-5.70
	2	1425	**1.70**	1.04-2.78	1.32	0.66-2.61	**2.15**	1.05-4.39
	3	2089	**2.53**	1.72-3.73	1.53	0.89-2.61	**4.30**	2.40-7.69
	4	661	**2.44**	1.19-5.02	1.92	0.61-6.08	**2.85**	1.10-7.37
MS	1d	1090	**1.53**	1.06-2.19	1.56	0.92-2.67	**1.69**	1.01-2.82
	2	725	1.53	0.98-2.42	1.94	0.97-3.86	1.58	0.84-2.99

Developing incident disorders in model 1: dyslipidemia, 73 men and 105 women; hypertension, 391 men and 395 women; diabetes, 90 men and 84 women; MS, 141 men and 143 women. Adjustments in model 1: sex, age; a: triglycerides, HDL cholesterol; b: waist circumference; c: waist circumference, fasting glucose; d: all 5 MS components. Model 2: apo B tertiles, additional to model 1a, c, and d. Smoking status in hypertension (1b). Model 3: sex, age, fasting glucose >100 mg/dL. Model 4: sex, age, log HOMA. Significant relative risks are highlighted in bold.

[a] Log-transformed values.

dL (RR, 2.53; 95% CI, 1.72-3.73; corresponding HR, 1.39; 95% CI, 1.21-1.59), although it was significant only in women (Table 4). In a subset of the cohort in whom HOMA index was available at baseline, CRP similarly predicted the development of diabetes in the whole sample and in women. Finally, among 999 subjects with known adiponectin concentrations at the final survey, baseline CRP predicted diabetes (RR, 3.30; 95% CI, 1.5-5.57) independent of the reciprocal association ($P = .038$) of adiponectin. Overall, 1.3% of subjects converted to diabetes per year; and Kaplan-Meier estimates in an up to 6-year follow-up showed that participants with CRP values ≥2.0 mg/L had significantly lower probability of remaining free of diabetes (89.6%) than those with <2.0 mg/L (95.0%, log rank $P < .0001$) (Fig. 1).

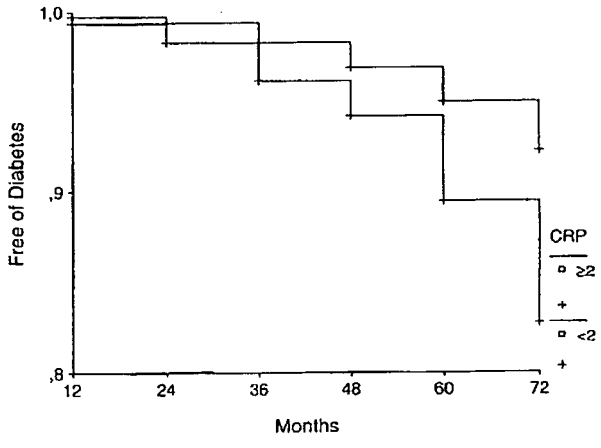

Fig. 1. Kaplan-Meier estimates of development of type 2 diabetes mellitus over 6-year follow-up, stratified by 1189 participants with normal (<2.0 mg/L) and 1109 with elevated (≥2.0 mg/L) CRP. A total of 60 and 116 subjects, respectively, were identified as having converted to diabetes.

3.5. Prediction of CHD

In a model comprising sex, age, waist girth, systolic BP, smoking status, fasting glucose, and HDL cholesterol in 2128 participants, log CRP predicted incident CHD independently in both sexes combined (RR, 1.61; 95% CI, 1.13-2.29; corresponding to an HR of 1.18; 95% CI, 1.04-1.34). Similar magnitude of associations in separate sexes remained at borderline significance (Table 5).

4. Discussion

In this prospective cohort study representative of Turkish adults, we found that smoking status, apo B levels, and low family income were major determinants of subsequently elevated CRP levels, albeit only in men. Increasing CRP levels significantly predicted in both sexes combined atherogenic dyslipidemia independently, subsequent hypertension irrespective of waist circumference, and subsequent

Table 5
Prediction of CHD by baseline serum CRP and certain traditional risk factors

	Total (n = 2128)		Men (n = 1017)		Women (n = 1111)	
	RR	*P*	RR	*P*	RR	*P*
Sex (female)	1.08	NS				
Age (y)	**1.06**	.0005	**1.055**	.0005	**1.063**	.0005
Waist circumference (cm)	1.012	.072	1.016	.13	1.011	.25
Systolic BP (mm Hg)	**1.015**	.0005	**1.016**	.002	**1.014**	.001
Fasting glucose (mg/dL)	**1.005**	.012	1.006	.052	1.005	.11
CRP[a]	**1.65**	.008	1.61	.069	1.57	.07
HDL cholesterol (mg/dL)	0.992	.21	1.000	NS	0.987	.12
Former smokers	1.40	.15	**1.86**	.028	0.73	NS
Current smokers	1.08	NS	1.46	.17	0.78	NS

One hundred nineteen men and 131 women developed incident CHD in follow-up. Significant relative risks are highlighted in bold.

[a] Log-transformed values.

MS, after adjustment for all 5 components. The CRP levels were a predictor of future diabetes in women regardless of baseline fasting glucose, waist circumference, and apo B, and of incident CHD in both sexes combined, independent of sex, age, and other relevant confounders. Thus, CRP levels were independently predictive of cardiometabolic risk, whereas sex, smoking habit, and apo B levels appeared to modulate the CRP-related risk.

To estimate the magnitude of the contribution of the risk involved with raised CRP levels, it should be kept in mind that the difference in median values between the top and the bottom quintiles, that is, between the 90th and the 10th percentiles, spanned a 15-fold gradient (7.5 vs 0.5 mg/L) in this population sample, like other populations. An RR of 1.7 across a gradient of 1 log CRP corresponds to an HR of 1.21, and an RR of 1.3 to an HR of 1.09 to 1.10 related to the outcome of individual cardiometabolic disorders. The contributed risk by the inflammatory component was modest when compared with HRs of waist circumference (2.02) or fasting triglyceride (1.88) derived from the identical multivariate model, but the risk was additive to that of the components or other risk factors and was of substantial magnitude particularly with respect to CHD.

This is the first documented study in which CRP predicted future MS, independent of age and the 5 components. Of the previous 3 prospective studies that did not fully adjust for the MS components, in the one on a Mexican population sample, with MS being defined in the absence of abdominal obesity, CRP was found to be a predictor in women but not in men [8]; and the reverse, namely, prediction of MS by rising CRP tertiles, was detected in men but not in women [13]. The risk of MS was several-fold higher with elevated CRP concentrations in the Finnish study that was confined to men [9].

4.1. Sex and smoking modify level and effect of CRP

Sex interacted in the predictors of elevated CRP levels and in the association of serum CRP with future diabetes risk after adjustment for age and relevant confounders. Current smoking, apo B levels, and family income were determinants of the inflammatory marker in men alone. Relationships of CRP levels with waist circumference, BP, and serum apo B, stronger in women than in men in univariate correlations (data not shown), were modified on multivariate analysis by smoking. Our findings in men are in line with the fact that cigarette smoking predicts inflammation [11,28]; associations with various markers of inflammation were generally lacking in women in the large cross-sectional multinational MONItoring of trends and determinants in CArdiovascular disease (MONICA) Augsburg study [28]. Whereas elevated CRP levels were significantly predicted by current smoking in men, a neutral effect was apparent in women, among whom age-adjusted current smokers exhibited even significantly lower CRP concentrations than never smokers. Lack of an induced inflammatory effect was remarkably independent not only of a marker of central obesity and fasting insulin, but also of family income, a surrogate of socioeconomic level.

We recently reported evidence that smoking protects Turks from obesity [25] and women from MS and diabetes [18], an effect which was independent of central obesity and hyperinsulinemia—and partly of serum CRP either. C-reactive protein is recognized to exert anti-inflammatory mechanisms, inasmuch as the direct interaction between CRP and complement can both activate and inhibit inflammation in atherosclerotic lesions [29]. The full reasons for this "protective" effect of smoking in Turkish women, an indication of which had previously been noted [30], remain unclear; but it seemingly results from a combination of reducing effects of smoking on abdominal obesity and on serum asymmetric dimethylarginine levels, lack of an inflammatory effect, and enhancement of serum preheparin lipoprotein mass (unpublished observations).

4.2. CRP, a risk factor for metabolic disorders, modified by apo B levels

Although prospective population-based data on the prediction of atherogenic dyslipidemia by CRP are lacking, 2 studies addressed the development of hypertension. In agreement with the positive relationships between baseline CRP and subsequent development of hypertension in women [12] and in men [11], CRP emerged as an independent predictor of hypertension in the present study. In the prediction of hypertension; high-triglyceride, low-HDL dyslipidemia; and MS, CRP, and apo B levels appeared to affect risk independently in men, whereas apo B and aging (menopause) were factors in women that mediated inflammation in the development of dyslipidemia. Independent of plasma glucose, CRP contributed significantly to the development of diabetes in women, additive to waist circumference and to apo B, whereas the potential influence of inflammation seemed minor in men. C-reactive protein contributed to new diabetes in women also regardless of a measure of insulin resistance or adiponectin; thus, it should not be considered merely a marker of fatness.

4.3. Independent predictive value of CRP for CHD risk

In predicting future CHD, the top CRP tertile (>3 mg/L) is expected to yield a mean multiple-adjusted RR of 2.0 according to a consensus Centers for Disease Control and Prevention/American Heart Association statement [31] or 1.45 in an updated meta-analysis [4]. Our estimated RR of 1.65 of log CRP is in close agreement with the cited meta-analysis because a CRP gradient across tertiles of the general population usually spans some 8-fold values.

Cardiometabolic risk is driven by adiposity (waist girth as marker), systemic inflammation (CRP), and small dense LDL (apo B), which are undoubtedly interrelated and related to insulin resistance. Based on this study and on separate analyses on apo B, waist girth appears to be dominant in regard to dyslipidemia, hypertension, and diabetes, with apo B or CRP, respectively, modulating the risk [17].

Development of CHD is driven by apo B, with CRP independently contributing in the risk. All 3 components are involved in the risk of MS independently. Smoking is likely an element in further modulating this risk on a sex-specific basis. Implications of this knowledge include the potential targeting of the treatment in various cardiometabolic disturbances in the future.

Like most similar prospective studies, single CRP measurements at baseline were used herein; but the weight of this potential limitation is relatively small in view of the recognized stability of this protein over a long follow-up [31]. The substitution of data of the subsequent survey for missing fasting measurement of serum triglycerides in a small proportion is not expected to substantially influence the results. Good agreement of and similarity between sexes in self-reported categories of smoking status render a potential substantial bias in female reporting highly unlikely. Unmeasurable confounding cannot be ruled out but is very unlikely in view of appropriate multiple adjustments performed. The strengths of the study include its being based on a representative sample of a general population, incorporation of women, prospective design and appropriate follow-up period, and measurement of studied parameters such as BP and plasma glucose.

We conclude that cigarette smoking, apo B levels, and family income are important determinants of serum CRP increments in the long term in Turkish men, independent of waist girth. Elevated CRP levels predict significantly future cardiometabolic risk pertaining to atherogenic dyslipidemia, hypertension, MS, diabetes, and incident CHD, independent of age, apo B, and other relevant confounders. These observations further support the concept of low-grade inflammation being critical in the development of the metabolic disturbances as well as of CHD. Sex and/or smoking habit is an important modifier of CRP concentrations; and serum apo B levels, by partly mediating CRP, modulate the cardiometabolic risk.

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